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BOVINE MASTITIS: A COMPARISON OF THE VALUE OF DIAGNOSTIC METHODS

By

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INTRODUCTION

Some workers have doubted the validity of using the results of cultural examination of milk samples as the standard by which to assess the value of indirect methods such as the chloride test, catalase test, Whiteside test, bromthymol blue test and total cell count in the diagnosis of mastitis. The aim of the investigation recorded here has been to obtain accurate information on this matter by comparing the changes produced in milk, as shown by indirect tests, with the results of bacteriological examination of the milk and the udder tissue itself, and with the histopathological changes present in the latter.

MATERIALS, TECHNIQUE AND PROCEDURE

Materials used in this investigation were obtained from farms near Edinburgh and in Ayrshire. Details of the history of all the cows were available except as regards seven, which came directly from the slaughterhouse and were kept for only a week whilst samples of their milk were being studied. The results of 87 quarters from 22 cows were recorded and are presented in this paper. Though two or three milk samples from each quarter were examined during the period of two to seven days before the animals were slaughtered, only the results obtained on the final samples, which were always collected within 24 hours of slaughter, are used here for comparison with the results of the histopathological examination.

The chloride test was carried out by direct titration, and milk which contained 0·14 per cent. or less of chloride was considered as normal. The catalase test was performed by adding one drop of hydrogen peroxide (9 vol.) to five drops of well-mixed milk in a watch glass. The reading was taken against electric light with the help of a hand lens at the end of five minutes. A negative result was not recorded unless there were no gas bubbles. For the Whiteside test the modified technique of Murphy and Hanson (1941) was employed; the appearance of strands or viscid masses at the end of 20 seconds was regarded as positive. For the bromthymol blue

* This paper is a part of the thesis approved by the University of Edinburgh in November, 1947, for the Ph.D. degree.

test, the method recommended by Stableforth (1930) was used. In estimating the total cell count, 1/500 ml. of well-mixed milk was transferred from a capillary measuring pipette to a clean slide and made into a uniform smear about the size of half a centimetre square. The smear was well dried in the air and stained by Newman's stain. All the cells were counted covering the whole smear, so that 500 times the number present would represent the total number in 1 ml. of the milk sample.

For the cultural tests on milk samples, both well-mixed fresh milk and samples incubated overnight were used. In all cases morphological and biochemical characteristics were employed for the identification of organisms : in addition, streptococci were examined serologically. For staphylococci the pathogenicity was determined entirely on the coagulase test using human blood plasma. Guinea-pigs were inoculated with the milk of abortion reactors but in no case was a strain of *Br. abortus* recovered.

All the cows were killed at the abattoir. The udders were taken to a laboratory for freezing and were then cut into slices $\frac{1}{2}$ to 1 inch thick. Three blocks of tissue from three of these slices representing the upper, the middle and the lower levels of the udder, were taken for culture tests after the surface of each had been sterilised by a hot iron. From adjacent slices four blocks were taken for histopathological examination, making twelve blocks in all from each udder. Sections were stained by haematoxylin and eosin, methylene blue and eosin, picro-Mallory's stain, Weigert's stain and Gram's stain.

Histopathologically, mastitis has been classified into five types : acute suppurative, acute catarrhal, subacute suppurative, subacute catarrhal and chronic. It should be emphasised, however, that as all five types not infrequently existed in the same quarter or even in the same section, each case was classified according to the predominating lesion present in the whole quarter as represented by the twelve blocks of udder tissue.

Quarters from each udder were identified as L.F. (left fore), L.H. (left hind), R.F. (right fore) and R.H. (right hind). The identification number of the cow from which the quarter was obtained precedes the letters.

RESULTS

Indirect Tests and Cultural Test

Tables I and II illustrate the relations of the indirect test to the culture test and histopathological examination respectively. The accuracy of the indirect tests was assessed in the following way. Taking the chloride test as an example (Table I), it was found that the results agreed with those of the culture test only in 36 positive and eight negative cases out of 87. Its accuracy was therefore 50·6 per cent. ; at the same time it was unreliable in 43 cases, comprising 40 culturally-negative quarters which it indicated to be positive or mastitic, and three culturally-positive ones returned as negative or non-mastitic. The total inaccuracy, including false positives and false negatives, was therefore 49·4 per cent. Similarly, the accuracy of the catalase test, Whiteside test, bromthymol blue test and total cell count, judged by the results of the culture test, was 46·0, 49·4, 55·2 and 45·0 per cent. respectively, each test showing a high percentage of false positives.

TABLE I

RELATIONS OF THE CHLORIDE TEST, CATALASE TEST, WHITESIDE TEST, BROMTHYMOL BLUE TEST AND TOTAL CELL COUNT TO THE CULTURE TEST OF 87 QUARTERS

Tests		†*Pathogenic organisms*		*Accuracy* (*per cent.*)	*Inaccuracy* (*per cent.*)	
		Absent in 48	*Present in* 39		*False* +	*False* —
Chloride test	0·14% or less	8	3	50·6	46·0	3·4
	More than 0·14%	40	36			
Catalase Test	Negative	4	3	46·0	50·5	3·5
	Positive	44	36			
Whiteside Test	Negative	10	6	49·4	43·6	7·0
	Positive	38	33			
Bromthymol blue Test	Normal	29	20	55·2	21·8	23·0
	Acidic or Alkaline	19	19			
*Total cell count	20,000/ml. or less	0	0	45·0	55·0	0·0
	More than 20,000/ml.	48	39			

* Total cell count : A figure of 20,000 cells per ml. of milk was taken as standard for normal milk, since pathological changes were revealed in one case (6LF) where the milk sample gave a figure of 21,000 cells per ml.

† The result was obtained from culture tests made on both fresh and incubated milk as well as udder tissue.

Table II shows that the accuracy of the chloride test, catalase test, Whiteside test and total cell count, when assessed upon histopathological examination, was much higher, being 87, 92, 82 and 100 per cent. respectively, but the accuracy of the culture test by similar comparison was only 45 per cent. The bromthymol blue test gave an accuracy of only 43·6 per cent. This suggests firstly that all the indirect tests, except the bromthymol blue test, are much more reliable than hitherto believed ; secondly, that the total cell count, when even as low a figure as 20,000 cells per ml., is taken as the borderline for normal milk, is so reliable that it can reveal mastitis conditions in 100 per cent. of cases, and finally that the culture test indicates only 45 per cent. of all mastitis cases.

A total cell count of 100,000 per ml., or even as high as 500,000 per ml., has long been regarded as normal, but the present investigation shows that of 39 culturally positive quarters, four had a total

cell count of 100,000 or less per ml., and twelve had a total cell count of 500,000 or less per ml. It also shows that out of 17 quarters with mastitis due to *Str. agalactiae*, 15 had a total cell count of 1,000,000 per ml. or more. Most of the Lancefield Group G. streptococcal cases which came from abortion reactors also gave a high cell count. Of all the 87 quarters showing histological lesions of mastitis, 13 had a total cell count of 100,000 per ml. or less, and as many as 40 had a total cell count of 500,000 per ml. or less.

TABLE II

RELATIONS OF THE CHLORIDE TEST, CATALASE TEST, WHITESIDE TEST, BROMTHYMOL BLUE TEST, TOTAL CELL COUNT AND CULTURE TEST TO THE HISTOPATHOLOGICAL EXAMINATION OF 87 QUARTERS

Tests		† *Pathological changes*		*Accuracy (per cent.)*	*Inaccuracy (per cent.)*	
		Absent in 0	*Present in* 87		*False +*	*False —*
Chloride Test	0·14% or less	0	11	87·0	0	13·0
	More than 0·14%	0	76			
Catalase Test	Negative	0	7	92·0	0	8·0
	Positive	0	80			
Whiteside Test	Negative	0	16	82·0	0	18·0
	Positive	0	71			
Bromthymol blue Test	Normal	0	49	43·6	0	56·4
	Acidic or Alkaline	0	38			
Total cell count	20,000/ml. or less	0	0	100·0	0	0
	More than 20,000/ml.	0	87			
* Culture Test	Negative	0	48	45·0	0	55·0
	Positive	0	39			

* † See footnotes of Table I

The differing results of the culture tests made from fresh milk, incubated milk and tissue of the udder from which the milk was collected, is worth noting. The total number of quarters infected with pathogenic organisms was 39, which represented 45 per cent. of all the mastitis cases. Culture tests made from fresh milk alone were positive in only 27 instances, from incubated milk alone in

only 34 and from udder tissue in only 30, taking as positive all cases in which the results from fresh and incubated milk were positive on one occasion and negative on another (represented by ±, Table III). In other words, in detecting mastitis the culture test gave correct results in 31 per cent. of all cases by using fresh milk, in 39 per cent. by using incubated milk, and in 34·5 per cent. by using udder tissue.

Regarding udder tissue cultures, most of the bacteriologically positive quarters were positive at one or two levels only (represented by ±). There was, however, no indication that tissues taken from the upper levels of the quarter were culturally negative more frequently than those from the lower, or *vice-versa*.

TABLE III

RESULTS OF THE CULTURE TESTS MADE FROM FRESH MILK, INCUBATED MILK AND UDDER TISSUE OF 39 QUARTERS

I	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*
39RF	+	+	+	835LH	+	+	+	43LF	—	±	+
39RH	+	+	+	835RF	+	+	+	43LH	+	+	+
438LF	+	+	—	835RH	+	+	+	43RF	—	±	—
438RF	+	+	±	136LH	—	—	±	43RH	+	+	+
438RH	+	+	+	136RF	—	+	—	73LF	+	+	±
517LF	+	+	±	136RH	—	—	±	7LF	—	—	±
517RH	+	+	±	132RF	—	+	±	7LH	—	—	±
562RH	+	+	+	132RH	—	+	±	7RH	—	+	±
680LF	+	+	+	1RF	+	+	+	38RF	+	+	—
680LH	±	±	—	2LH	+	+	+	42LF	+	+	—
680RF	±	±	—	2RF	+	+	+	42LH	—	+	—
680RH	+	+	+	29LF	+	+	+	42RF	+	+	—
835LF	+	+	+	3LF	+	+	+	42RH	—	—	±

I = Quarters. II = Fresh Milk. III = Incubated Milk. IV = Udder Tissue.

Staphylococci which did not coagulate human blood were often encountered in milk alone or in milk and udder tissue ; 21 strains were isolated, of which 14 were obtained from both sources and the remainder only from the milk. All except four invariably fermented mannitol in from one to seven days ; some were haemolytic, or weakly so, but few of them gave golden yellow pigmentation.

Histopathological Examinations

It has been found that in normal, fully lactating udders, the interalveolar connective tissues are reduced to a minimum. With the completion of the lactation period, the alveoli became smaller and smaller due to decrease in size of their cells, and gradually disappeared through degeneration and disintegration, leaving only ductules and a few surviving alveoli with very loose connective tissue between them. Even at this stage the intralobular connective tissue was by no means prominent, fatty tissue being normally present. The author does not agree with Gibbons (1938) that during the dry period the epithelium of the alveoli becomes massed into a

double row of cells, for it was found that in the normally regressing udder the persisting ductules and alveoli always maintained a regular, single-celled layer of epithelium. In some cases of mastitis showing an increase of connective tissue the alveoli were shrunken and folded and appeared very cellular.

In normal udders, lymphocytes are usually present in small numbers, but polymorphonuclear leucocytes are rare (Petersen, Hastings and Hadley, 1938; McFarlane, Rennie and Blackburn, 1947). The latter, if at all numerous, should be regarded as an indication of inflammatory change, while appreciable numbers of macrophages, plasma cells, and eosinophiles are considered as evidence of a chronic inflammatory process.

Before describing the pathological changes, the author wishes to emphasise again that he has classified his cases entirely according to the type of lesion predominating in each, and that other types of changes were also present in the udder at the same time.

Acute Suppurative Mastitis.—The main features were presence of pus, infiltration of polymorphonuclear leucocytes, shedding and destruction of epithelial cells. The changes were progressive, and fibrosis entirely absent, or at most present to an insignificant degree. However, no quarter was found to show such changes predominantly, although they were present in some cases of subacute suppurative and subacute catarrhal mastitis.

Acute Catarrhal Mastitis.—The picture was much the same as in acute suppurative mastitis except that there was no suppuration. Eleven quarters were found to belong to this group; of these four were infected with *Str. agalactiae*, one with pathogenic staphylococci, and six showed no pathogenic organisms.

Subacute Suppurative Mastitis.—The changes were the same as in acute suppurative mastitis and were progressive except that productive or fibrotic changes were in evidence. Five quarters fell into this category, four being infected with *Str. agalactiae* and one with *Corynebact. pyogenes.*

Subacute Catarrhal Mastitis.—The changes resembled those of acute catarrhal mastitis except that productive or fibrotic changes were more pronounced. There were 33 quarters in this group, of which nine were infected with *Str. agalactiae*, one with *Str. dysgalactiae*, three with *Str. uberis*, seven with Lancefield Group G. streptococci, two with pathogenic staphylococci and eleven yielded no pathogenic organisms.

Chronic Mastitis.—This term was applied where changes were predominantly productive, characterised by an increase of fibrous tissue, infiltration of histiocytes, lymphocytes, eosinophiles, shedding and destruction of epithelial cells and shrinkage of alveoli. Thirty-eight quarters were classified as having chronic mastitis, of which three were infected with *Str. dysgalactiae*, two with *Str. uberis*, one with Lancefield Group G. streptococci, one with pathogenic staphylococci, and there were 31 from which no pathogenic organisms were isolated.

DISCUSSION

Accuracy of the Culture Test for Identifying Mastitis.—According to the present investigation, of the 87 mastitic quarters diagnosed by histological examination, only 39 could be identified by the culture test, although this was carried out on both fresh and incubated milk samples as well as udder tissue. The number of positive cases would have been even less had cultures been made from only one of these, *i.e.*, 27 positive cultures from fresh milk, 34 from incubated milk and 30 from udder tissue.

However, the culture test cannot be expected to agree with the histopathological examination in every case. On the one hand, elimination of organisms in bacterial mastitis may be intermittent (Davis, McClemont and Rogers, 1939; Udall, 1947a; Garside, 1947), and this is supported by the present work. Consequently, the causal organisms, although present in the udder, cannot invariably be isolated therefrom. Further, they may disappear from the udder as a result of the antibacterial action of the tissues or because of too high an acidity of the milk created by bacterial action. Again, in the early stages of mastitis where the udder shows only a mild, acute inflammatory reaction, the number of organisms present might be too small to be detected, even after nine attempts at culture. On the other hand, inflammation of the udder might be due to incomplete milking, rough manipulation or faulty byre construction, as suggested by Udall (1947b). Morris (1944) reported a leucocytosis in the blood during the drying period and also in the two weeks before parturition. The presence of mild infiltration of polymorphonuclear leucocytes in some cases might thus be the result of physiological change in the blood. In any case it would appear from the present work that the culture test is not the most effective means for detecting mastitis and it cannot, therefore, be used as a basis for assessing the diagnostic value of the indirect tests.

Accuracy of Indirect Tests for Identifying Mastitis.—As judged by the histopathological examination, the accuracy of the indirect methods, with the exception of the bromthymol blue test, was found to be much better than hitherto thought. Theoretically, any deviation from normal milk should be indicated by the indirect tests, because in the presence of inflammatory reaction in the udder, the milk shows excess of chloride, change of *p*H and the influx of small or large numbers of inflammatory cells. Each test will be discussed with reference to variations in both normal and abnormal values.

Chloride Test.—It is known that milk collected from the teat is, as a rule, a mixture of secretion from all parts of the quarter, so that the chloride content of the milk from an area of inflammation, though higher than normal, may be levelled down when the milk mixes in the sinus with the secretion from normal parts of the gland; this is particularly true during the early part of lactation when, according to Caulfield and Riddell (1935), the normal chloride content is well below the usual 0·14 per cent. Many other factors,

such as breed, age, number of lactations and seasonal effects also influence the chloride content of the milk. The test, therefore, serves as a useful indicator only if all such factors are taken into consideration.

Catalase Test.—The author does not agree with Tompkins, Plastridge and Bryan (1946) in their statement that : " It is generally agreed that this test may be too sensitive to serve as a basis for classifying cows affected with mastitis." In the present investigation it was found that all milk samples from mastitis quarters having a total cell count of less than 155,000 failed to react positively to the test. However, this failure may not be entirely attributable to the test itself, since high acidity (Klein, 1917) and cold storage may inhibit the activity of catalase.

Whiteside Test.—This was found to be less sensitive than the catalase test, its accuracy being only 82 per cent. (Table II). The results might have been improved had readings been made at the end of 30 seconds instead of 20 seconds, which was the time-limit set by Murphy and Hanson (1941), since quite often the viscid material did not appear until after 20 to 30 seconds had elapsed. Moreover, some of the milk samples had to be kept in the refrigerator for 12 to 14 hours before testing, and although different amounts of NaOH solution were used, as recommended by Murphy, the results may not by strictly comparable with those given by fresh milk, or *vice-versa*.

Bromthymol Blue Test.—This test was of little value in detecting mastitis, its accuracy being only 43·6 per cent. (Table II). It may be that early mastitis is not detectable by this test because the alkalinity of the secretion from the area of mastitis is reduced by mixing with secretion from the normal part, and also because bromthymol blue itself is relatively insensitive to slight changes in *p*H. Further, the alkaline reaction of the milk, which as a rule results from changes in the permeability of the cells, may be neutralised by acid produced from lactose by the action of the organisms. If the acid is in excess, the milk will give from slight to frank acid reaction. This explains why Zeich (1937) found that milk from cows with streptococcal mastitis had a *p*H of about 6·0, which in chronic cases might drop to about 5·2. Finally, according to McDowall (1941), reading of the test is affected by the fat content of the sample, and the higher this is the more difficult is it to detect the blue colour which indicates a positive result.

Total Cell Count.—This method is clearly the most effective indirect test of all as judged by the histopathological examination. In this work, whenever the total cell count was above 20,000/ml., inflammatory changes could be found in the udder. Therefore, a figure of 500,000 or even one as low as 100,000 per ml. suggested by many workers as the borderline for normal milk, can no longer be accepted. But adequate data as to the maximum number of cells that may be found in normal milk are not yet available, and the figure of 20,000 per ml. was provisionally adopted, though with

some hesitation, as the borderline for normal milk. It is appreciated, therefore, that this test may give a number of false positives when interpreted on this basis.

Accuracy of Culture Test versus *that of Indirect Tests for Identifying Mastitis.*—In nearly all previous work indirect tests have been shown to be of little diagnostic value owing to the fact that the culture test was there used as the standard of evaluation. It is now clear that certain of these tests, such as chloride test, catalase test, Whiteside test and total cell count, are very useful in detecting inflammation irrespective of aetiology. It is also evident that the culture test does not reveal all cases of mastitis and those cases in which the pathogenic organism is absent but inflammation is present will be classified as false positive, thus reflecting erroneously upon the value of the indirect test. However, it is generally agreed that from the economic point of view cases of mastitis which yield pathogenic organisms are the most significant. Thus, although the indirect tests were found to be much more in agreement with the histopathological findings than the culture test, the latter remains the most important diagnostic procedure when combating mastitis by modern therapeutic methods. Nevertheless, the routine herd test performed only once every few months will not always give the correct result since the present work has shown a great deal of variation in the results given by the milk samples collected at different times or by udder tissue taken from different levels. It follows, therefore, that for accurate diagnosis of " bacterial mastitis " it is advisable that the culture test should be carried out frequently and regularly.

SUMMARY

A study of 87 quarters from 26 cows affected with mastitis was made in order to correlate the results given by five indirect tests and the culture test of milk samples with those given by the cultural and histopathological examination of the tissue of the udder from which the milk was collected.

The accuracy of the chloride test, catalase test, Whiteside test and the total cell count was found to be 50·6, 46·0, 49·4 and 45·0 per cent. respectively, when judged on the basis of the culture test as was customary with previous workers. When assessment was made on the results of histopathological examination of the udder tissue, the comparison was 87 per cent. for the chloride test, 92 per cent. for the catalase test, 82 per cent. for the Whiteside test, and 100 per cent. for the total cell count. The bromthymol blue test was found to be unreliable.

The culture test, even when using both fresh and incubated milk as well as udder tissue, was not capable of identifying more than 45 per cent. of all mastitis cases, as judged by histopathological examination of the udder tissue.

A total cell count of 100,000 per ml. which has been generally regarded as the borderline for normal milk, was shown to be too

high. It was found that pathological changes were present in the udder tissue whenever the count was above 21,000 per ml.

The pathological changes caused by *Str. agalactiae* were found to be primarily subacute catarrhal, marked by highly productive changes in the interstitial tissue and severe leucocytic infiltration in the alveoli, while those caused by *Str. dysgalactiae*, *Str. uberis*, and pathogenic staphylococci were much milder. Although quite intensive lesions were also produced by *Str. uberis*, they were much less severe than those caused by *Str. agalactiae*. Lesions observed in one case of *Corynebact. pyogenes* infection were mainly severe, subacute suppurative, characterised by organisation of the suppurative exudate. The fibrous tissue, thus formed, remained resulting in extensive fibrosis.

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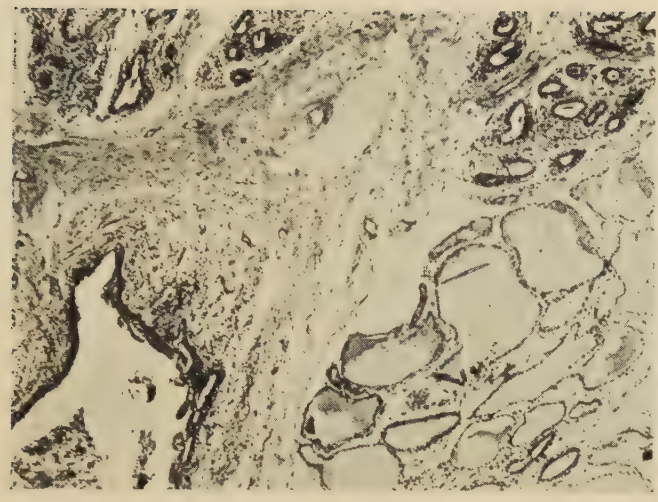

FIG. 1.—The lobule on the right lower side apparently normal; elsewhere showing shrinkage of alveoli, increase of interalveolar connective tissue, infiltration of lymphocytes, macrophages, and eosinophiles; in the duct were cells and streptococci; *Str. agalactiae* isolated.

(835 LH 3.) Haematoxylin and Eosin. × 80 diam.

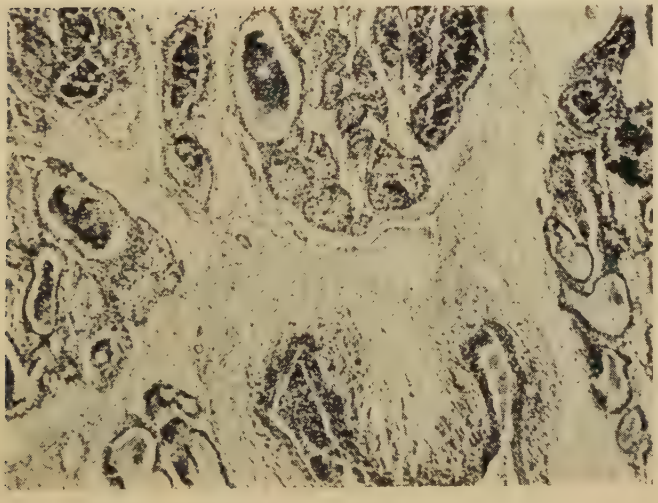

FIG. 2.—Infiltration of polymorphs and shed epithelial cells or nuclei in the alveoli and ducts; *Str. agalactiae* isolated.

(438 RF 2.) Haematoxylin and Eosin. × 80 diam.

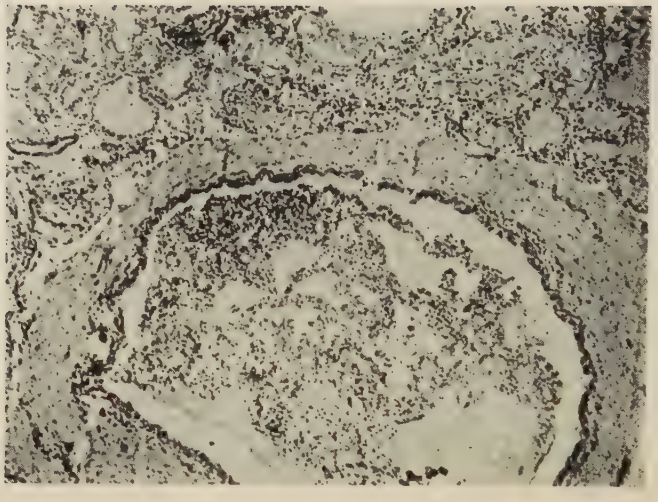

Fig. 3.—Lymphocytic foci in upper part of the picture; a suppurative focus in the dilatated ductule, note the thickened wall; *C. pyogenes* isolated.

(43 LH 1.) Haematoxylin and Eosin. × 80 diam.

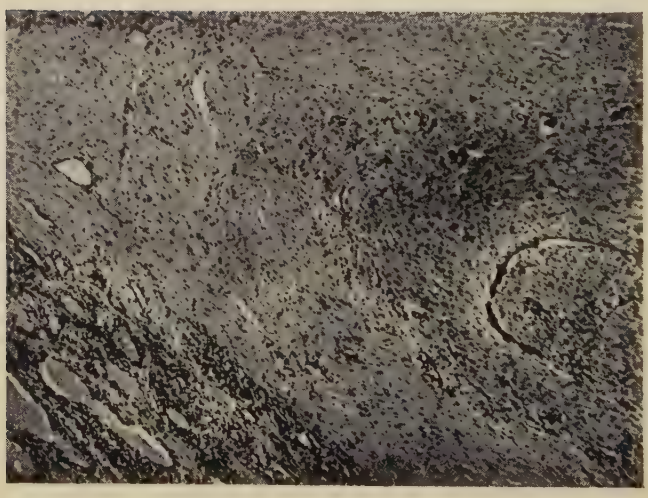

Fig. 4.—Alveoli obliterated and replaced by fibrous tissue; a ductule on the right side obstructed by fibrous tissue; *C. pyogenes* isolated.

(43 LH 3.) Haematoxylin and Eosin. × 80 diam.

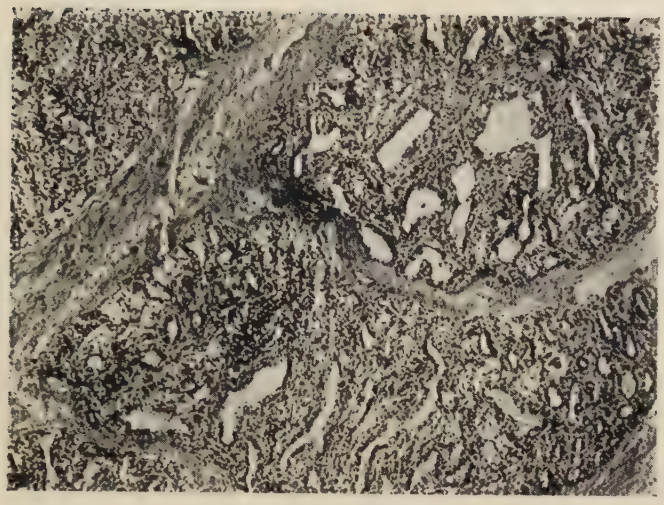

FIG. 5.—Shrinkage of alveoli ; increase of interalveolar connective tissue with lymphocytic infiltrations ; Group G. streptococci isolated.

(43 RH 3.) Haematoxylin and Eosin. × 80 diam.

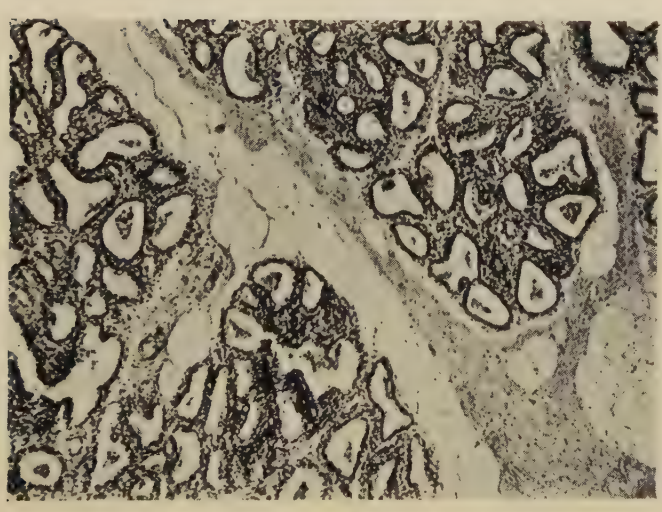

FIG. 6.—Infiltration of eosinophiles in the connective tissue ; shrinkage of alveoli ; shedding of epithelial cells ; lymphocytic infiltrations ; no pathogenic organism isolated ; cow showing positive reaction to abortion test.

(38 LH 2.) Haematoxylin and Eosin. × 80 diam.

A CASE OF GENERALISED AMYLOIDOSIS IN THE DOG

By

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INTRODUCTION

References to Amyloidosis in domesticated animals are extremely scanty in English veterinary literature. Most of the naturally occurring cases reported have been in horses and its occurrence in those used for the production of antitoxic sera is well known. The Continental literature contains a number of papers on amyloidosis in the dog, but no case appears to have been described in this country; the present account, therefore, may be of interest.

DESCRIPTION OF CASE

The affected animal was a 13½-year-old male golden cocker spaniel which had a history, extending over some years, of prostatic enlargement the clinical symptoms of which had been relieved by stilboestrol. Parodontal disease had been present for several months at least and had rapidly progressed shortly before death. Eleven weeks before it died the dog had lost a lot of flesh, and its breath smelt strongly uraemic. The urine at that time had a specific gravity of 1,016, showed albumin (+ + + +) with the salicylsulphonic acid test and contained small quantities of bile pigment. There was little thirst and no loss of appetite. This clinical picture obtained until three days before death when a rapid decline in general condition became evident. Uraemia was by now intense. The animal was destroyed on 31.3.48. At the time of death, agglutinins for *Leptospira canicola* were present in the serum to a titre of 1 : 300, but none for *L. icterohaemorrhagiae* were found.

Blood obtained at autopsy four hours after death showed a blood urea level of 156 mg. per cent. (Urease method).

The serum protein levels were albumin, 2·21 per cent.; globulin, 3·29 per cent. and the A/G ratio 0·67 : 1 (Howe's method).

Autopsy Findings.

Buccal Cavity.—The teeth showed heavy calculus deposits associated with a suppurative gingivitis and parodontal disease. No ulceration of the tongue or buccal mucosa was present. Slight enlargement but no congestion of the submaxillary lymph gland was evident.

Respiratory System.—The trachea, bronchi, and lungs, bronchial and mediastinal lymph glands were macroscopically normal.

Cardiovascular System.—A slight excess of fluid was present in the pericardial sac. The cusps of the mitral valve showed a moderate degree of nodular thickening and wrinkling but the valve was competent. The other valves were normal and the heart itself showed neither dilatation nor hypertrophy. The myocardium was of normal colour and consistency. The heart weight : body weight ratio was 0·009367 which falls within the accepted

normal limits. After separating the ventricles by Herrmann's method, the left ventricular : right ventricular weight ratio was 1·542 : 1. From the figures given for normal dogs (Herrmann, 1926), neither of these ratios can be regarded as convincing evidence that the heart showed either general or local hypertrophy.

The aorta showed a moderate degree of intimal thickening in the arch and a marked degree in the posterior abdominal portion. No abnormality was evident in the pulmonary trunk.

Alimentary Canal.—The oesophagus and stomach showed no abnormality. The duodenal mucosa was profusely spattered with petechiae and ecchymoses, and an occasional haemorrhage was present in the jejenum, ileum and large intestine. The mesenteric lymph gland showed no abnormality. The liver, gall bladder, bile ducts, pancreas and spleen showed no macroscopic change.

Urogenital System.—Both kidneys were reduced in size but not grossly so. A speckled appearance of the underlying cortex was apparent through the capsules ; these were stripped with difficulty leaving a somewhat granular surface over which were distributed numerous white points representing intact groups of tubules rendered more distinct by contrast with the surrounding fibrous tissue. On cutting, the kidney was notably firm. The normal cortico-medullary pattern and proportions were preserved. On treatment with iodine solution followed by strong sulphuric acid, the glomeruli stood out as minute black points from the surrounding unstained tissue. The bladder showed a low grade cystitis. The prostate showed no significant hypertrophy.

Endocrine Glands.—The parathyroids were slightly enlarged, but the thyroids and adrenals showed no change. The pituitary was not examined.

C.N.S.—Was not examined.

There was no evidence of active or healed tuberculosis.

Material was taken for microscopical examination from the kidney, lungs, liver, myocardium, pancreas, spleen, pectoral muscle, prostate, adrenal, a lymph node near the pancreas, the aorta (at the commencement of the arch and from the anterior abdominal region), right and left renal arteries, coeliac artery and anterior mesenteric artery. Paraffin sections were stained with haematoxylin and eosin, van Gieson and Weigert's elastic stain, Gram's stain, von Kossa's method, with methyl violet and with Congo Red. Frozen sections were stained with Scharlach R.

Microscopical changes

Kidney.—The structures of all the glomerular tufts was completely replaced by considerable masses of amyloid in which only a small number of endothelial cell nuclei were still present. All the glomeruli were swollen. The mean capsular diameter was 229μ as compared with the mean capsular diameter of a normal ten-year-old female spaniel which was 199μ ; the difference is statistically significant. In some cases the tufts filled the capsular spaces fairly completely ; in others there was dilatation of Bowman's capsule. Both this space and the lumen of the proximal convoluted tubule were filled with hyaline eosinophil material which failed to stain metachromatically

with methyl violet and had the appearance of protein. As the process was far advanced it was not possible to determine the site at which amyloid was first deposited in the glomerular tufts. Well marked pericapsular fibrosis was present in many glomeruli and others showed varying stages towards complete hyalinisation; occasional tufts still showed traces of amyloid in spite of being almost completely replaced by fibrous tissue. Shrinkage of the kidney as a whole was evidenced by the close proximity of the glomeruli to one another.

The majority of the tubules contained the hyaline material noted above. In many tubules the lumen was greatly dilated, the lining epithelium and its nuclei were flattened and the nuclear chromatin showed condensation. In other tubules the lumen was still dilated but the lining cells were less flattened and showed infiltration with minute droplets of fat: this fat was isotropic. In many tubules, particularly those lying near the capsule and showing atrophic changes, the cytoplasm of the lining epithelium cells showed granules staining by von Kossa's method and having a special predilection for the basement membrane of these tubules which in von Kossa-stained preparations were outlined as black rings. Many cortical tubules, particularly those near the capsule, showed advanced atrophy and distortion, disappearance of the lining epithelium and occlusion of the lumen by granular amorphous casts. The deposition of mineral material appeared to be the main cause of the epithelial desquamation in this group.

All the medullary tubules showed cystic dilatations and as the renal pelvis was approached the lining epithelium showed metaplasia to a multi-layered type resembling transitional epithelium.

Both cortical and medullary interstitial tissue showed a marked increase in fibrous tissue most pronounced near the boundary zone and renal pelvis and a moderately severe infiltration with lymphocytes and plasma cells. No giant cells or other evidence of resorptive phenomena were present.

A small number of arterioles near the boundary zone showed a moderate degree of intimal fibrous hyperplasia. In some cases this had caused a significant encroachment upon the lumen of the vessel. No changes were present in the larger intrarenal vessels.

Amyloid was present (1) in all glomerular tufts, whether functional or partially hyalinised, and occasionally in the periglomerular capsule; (2) in the cortex where it was found in the walls of the small arterioles either under the intima or throughout the wall irrespective of any particular coat although there was on the whole a tendency for the tunica adventitia to be affected in preference to other coats; (3) in the walls of the intertubular capillaries of the medulla; (4) in small deposits in both cortical and medullary interstitial tissue. The deposits in all these sites except the first were inconspicuous. The amyloid stained well with iodine and sulphuric

acid and showed typical metachromatic staining with methyl violet.

Prostate.—There was a marked glandular involution with increased interacinar fibrous tissue. Several foci of mild, round-celled infiltration were present. Amyloid was found in the walls of arterioles and of small venules, in small masses in the connective tissue of the gland, between muscle fibres and occasionally in the walls of prostatic acini.

Spleen.—The spleen showed severe hyaline changes in the intima and media of the arterioles. Amyloid was present in the walls of the small arterioles, either throughout the whole thickness or merely in the tunica adventitia. Amyloid was also present in small masses in the pulp and between the muscle fibres of the trabeculae.

Aorta.—At the commencement of the aorta the outer part of the tunica media showed several foci of medial necrosis with fibrous replacement of the type described by Morehead and Little (1945). The tunica intima exhibited intimal thickening of fibrous and elastic tissue. A few small deposits of amyloid were present in the tunica media.

The anterior abdominal region showed a thick intimal proliferation of collagen and elastic tissue of the usual type (Morehead and Little, 1945) and in parts some duplication of the internal elastic lamina. Amyloid was present in the form of a row of small deposits in the depth of the intimal thickening, just internal to the internal elastic lamina.

No significant histological change was present in sections of the pancreas, liver, lungs, myocardium, pancreatic lymph node and pectoral muscle stained by haematoxylin and eosin. Amyloid was present only in the small arterioles of the pancreatic lymph node and as a small deposit in the wall of a vein in the lungs but was absent from the remainder of the above mentioned organs. No amyloid was present in the renal, anterior mesenteric or coeliac arteries.

DISCUSSION

Amyloidosis in dogs is undoubtedly of infrequent occurrence, though Continental authors differ somewhat in their opinions on this point and the true incidence of the condition is difficult to assess. Hjärre (1933) encountered eleven cases in ten years among 1,700 autopsies, but Hissbach (cited by Hjärre) apparently met none among 500 autopsies. The recorded cases fall into two groups the larger of which includes those with no recognisable cause and the smaller those associated with chronic suppurative conditions or tuberculosis. This preponderance of idiopathic cases is the reverse of that found in man in whom tuberculosis is now the most frequent underlying cause. However, the number of recorded cases in dogs is comparatively small and the relative proportions for the two groups may have no statistical significance.

Aetiologically, the association of amyloid with tuberculosis and long-standing suppurative lesions in man and also its experimental occurrence in horses used for serum production, in mice injected with sodium caseinate, in rabbits following injections of staphylococci, etc., all suggest an association with antibody production as one of the factors in its causation. A number of cases in man have been secondary to multiple myelomatosis which is often accompanied by a hyperglobulinaemia.

Viewed against this background, the observations upon the serum protein levels in the affected dog become of interest. In the present instance there had been heavy and continuous loss of protein in the urine for over three months at least. In most forms of albuminuria, the plasma protein which most readily escapes through the glomerular filter and appears in the urine, is plasma albumin with its comparatively low molecular weight. In renal amyloidosis in man, in whom the glomerular lesions are histologically identical with those in the present case, the impairment of glomerular efficiency is sometimes sufficiently great to permit the escape also of the much larger molecule of plasma globulin, so that in late cases a reversal of the normal A:G ratio in the plasma and urine may occur. No figures are available in the present case for the relative proportions of the two fractions in the urine, but it would seem very possible that considerable loss of plasma globulin had occurred. If that were so, the serum globulin figure for the sample taken at autopsy from the affected dog may have been well below the concentration present when amyloid first became deposited. As they stand, the serum protein levels at the time of death are abnormal, th ealbumin being low and the globulin close to, if not slightly outside, the upper extreme of the normal range. Authors are not unanimous as to what should be regarded as the normal upper limit of plasma globulin in the dog (Allison *et al.*, 1941 ; Matthew, 1927 ; Weech *et al.*, 1935). The A:G ratio is very low.

The changes in the kidney are strikingly similar to one of the two types of renal lesion described by Hjärre (loc. cit.) in eleven cases of canine amyloidosis. The clinical symptoms are also very similar, particularly the low specific gravity of urine, very heavy albuminuria and uraemia. Whether this case should be regarded as one of secondary contracted amyloid kidney, or whether the amyloid has been deposited in a kidney already showing the changes of chronic canine nephritis, is difficult to say ; it is evident, however, that apart from the amyloid deposition, many of the changes are indistinguishable from those found in the late stages of chronic canine nephritis unassociated with amyloidosis.

There is one other point worth noting. The amyloid deposits in all the affected organs other than the kidney were quite inconspicuous in sections stained by haematoxylin and eosin, and were detected only by special staining methods the use of which was

prompted by the demonstration of very striking amyloid changes in the kidney. This might be of significance in connection with some of the reported cases where apparently the kidney was the only site of amyloid deposition.

SUMMARY

A case is described of amyloidosis associated with severe suppurative gingivitis in the dog. Amyloid deposits were present in the renal glomerular tufts and in the small vessels and interstitial tissue of the kidney and other organs.

A depleted serum albumin and a somewhat raised serum globulin level was noted in blood taken shortly after death.

ACKNOWLEDGMENTS

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I am deeply indebted to Miss J. O. Joshua, M.R.C.V.S., for the history and clinical details of the case.

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Fig. 1

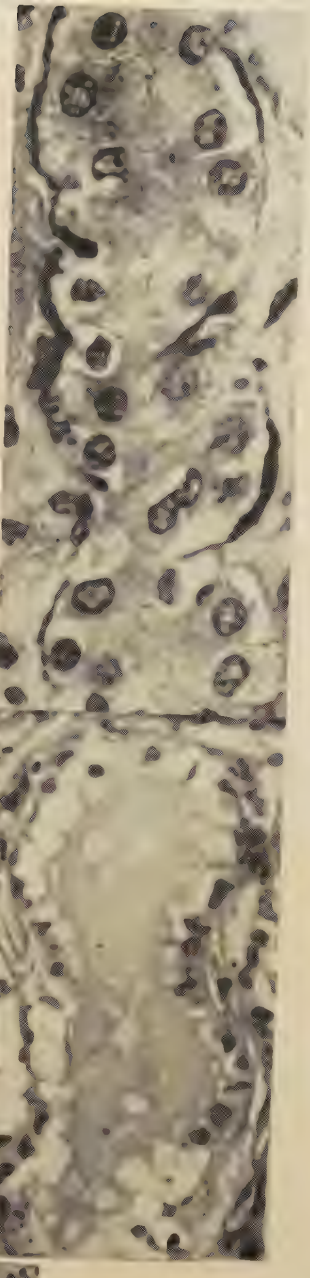

Fig. 3

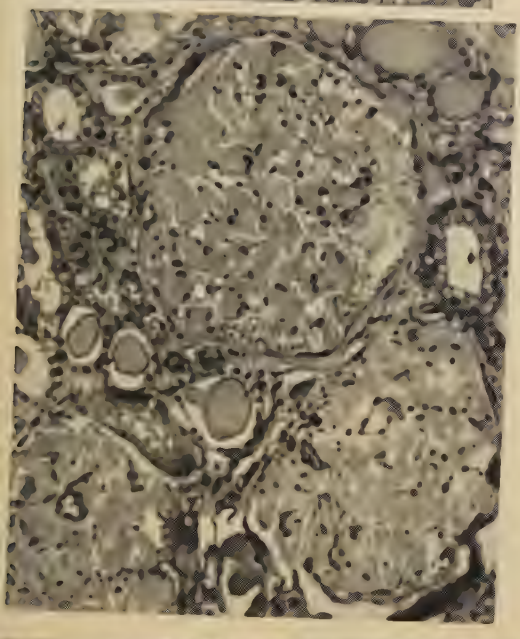

Fig. 2

Fig. 4

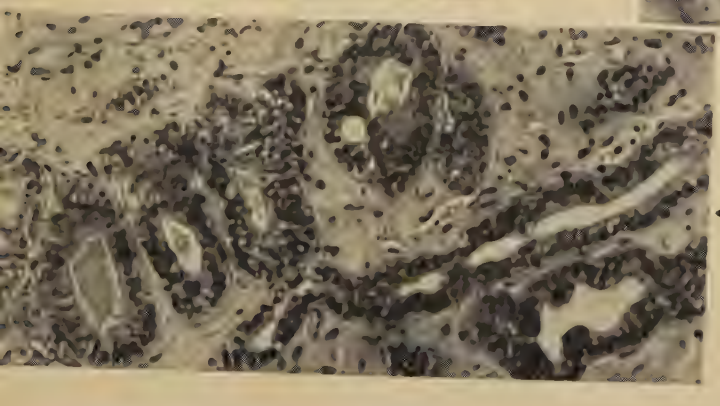

Fig. 5

FIG. 1.—General view of cortex of affected kidney. × 22. van Giesen and Weigert elastic stain.

FIG. 2.—Renal glomeruli. × 170. H. and E. stain.

FIG. 3.—Convoluted kidney tubule showing deposition in the basement membrane of material staining by von Kossa's method. 700. H. and E. stain.

FIG. 4.—Convoluted kidney [illegible]

INFERTILITY IN CATTLE ASSOCIATED WITH DEATH OF OVA AT EARLY STAGES AFTER FERTILISATION

By

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INTRODUCTION

Infertility in cattle can arise for a number of reasons. Infectious diseases, the use of infertile bulls, anatomical abnormalities and ovarian disfunction are common causes, but in this country a type of infertility often occurs as a herd condition, in which no abnormality has been detected by clinical or *post-mortem* examination. This type of infertility is often characterised by irregular oestrous cycles, and while it occurs at all seasons of the year, it is most frequent during the winter months. There is evidence that in summer this so-called "featureless" infertility results from service too early in the oestrous period for the spermatozoa to survive till ovulation occurs (Trimberger and Davis, 1943; Laing, 1945a). But this cannot be the sole explanation, for in most animals the oestrous period is too short for this to be a common cause even in the summer months. It was found (Laing, 1945b) that fertilisation always followed when sufficient spermatozoa were deposited in the uterus within sixteen hours before the end of oestrus and ovulation occurred at the normal time. Frequently, however, some fertilised ova developed for a time and then died, and this seemed to indicate that early death of the fertilised ovum might be a possible cause of infertility in cases where no abnormality could be demonstrated in the genital tract.

The following experiments were designed to obtain further information on this aspect of the infertility problem.

MATERIAL AND METHODS

The animals used consisted of two groups, H and J, each of 24 Shorthorn heifers. The history of Group H prior to the experiment is as follows. Heifers 1, 2, 3, 5, 6, 7, 8 and 10 were run with the bull from January to March, 1944: they failed to conceive and were fattened. Numbers 9, 13, 15, 16, 19, 20, 21, 24, 25, 27, 28, 35 and 38 were maiden heifers before the start of the experiment. H22 was inseminated three times, failed to conceive and was then fattened; H34 was served twice, failed to conceive and was then fattened. No history was available for H23. Group J was selected from a larger group of about 100 maiden heifers on the basis of normal physical condition and normality of the genitalia so far as is detectable by clinical examination per rectum and per vaginam. They were negative to the tuberculin test and to the agglutination test for

Br. abortus infection. In both groups the ages of the animals varied between 20 months and two years.

Group H.—The 24 heifers were kept in open yards during the months of May, June and July and fed a ration of 3 lb. of mixed concentrates and unrestricted meadow hay. At the beginning of the experiment they were examined by rectal palpation and vaginal inspection with a speculum to detect any abnormality of the genital tract. At eight-hour intervals after this (7.0 a.m., 3.0 p.m. and 11.0 p.m.) a fertile bull was put with them and any signs of oestrus noted. Any heifers which were in season were served. They were also inseminated with 1 c.c. of high density semen showing wave motion collected from a bull of known high fertility. The semen was deposited in the anterior cervix or body of the uterus by rectal manipulation of a glass pipette. Insemination was timed to the nearest quarter of an hour and performed within 30 minutes of collection of the semen. Oestrus was considered to commence half-way between the last occasion on which a heifer was not served and the first occasion on which she was served, and to end midway between the last time at which an animal was served and the first time at which she was not served. All animals were examined per rectum within 25·5 hours of the end of heat to determine whether ovulation had occurred. If the ovaries were smooth with no follicles palpable, it was concluded that ovulation had occurred. Examination was timed to the nearest quarter of an hour.

Six heifers, selected at random and spaced throughout the experiment, were killed as controls at various intervals from 40.00 to 66.75 hours after the end of oestrus (Table II). Slaughter was timed to the nearest quarter of an hour. The ova were recovered from the Fallopian tubes by the method of Heuser and Streeter (1929), examined and photographed.

The remaining 18 animals were "tried" with the bull every eight hours after the end of oestrus. They were examined per rectum at weekly intervals to follow the changes in the ovaries. Those which did not return at the first expected oestrus period were killed at times between 25 and 27 days after the end of oestrus. Those which returned to oestrus were re-served, re-inseminated and examined as before, killed within 72 hours of the end of oestrus and the ova recovered from the Fallopian tubes. Accuracy of timing was as before.

The ova, portions of uterus and corpora lutea and the embryos were fixed in Bouin's fluid, embedded and sections cut for histological examination.

Group J.—The experiment of the previous year was repeated with Group J in the next spring and summer, except that the heifers were examined to detect ovulation at 20 hours *post-oestrum*, and all were allowed to complete pregnancies or if they returned to oestrus were re-inseminated at successive periods. Those which did not become pregnant were killed later and examined *post-mortem*.

RESULTS

Group H

The time of insemination varied from 15·75 hours before to 1·5 hours after the end of oestrus (Table I). Examinations per rectum to detect whether ovulation had occurred were made at times varying from 21·0 to 25·5 hours after the end of oestrus (Table I.). In every case it was considered that ovulation had occurred before examination.

TABLE I

INSEMINATION IN RELATION TO OESTRUS (GROUP H)

No.	*Time of insemination.*	*Time of examination for ovulation*
H.1	11.5 hours before the end of oestrus	23·25 hours after the end of oestrus
H.3	0.25 „ „ „ „	22·25 „ „ „ „
H.7	0·5 „ after „ „	24·5 „ „ „ „
H.8	0·5 „ before „ „	24·0 „ „ „ „
H.16	1·5 „ after „ „	25·5 „ „ „ „
H.19	4·0 „ before „ „	21·0 „ „ „ „
H.27	7·75 „ „ „ „	21·0 „ „ „ „
H.28	0.5 „ after „ „	22·5 „ „ „ „
H.38	0.25 „ „ „ „	
	2·0 „ „ „ „	22·75 „ „ „ „
H.2	0·25 „ „ „ „	24·00 „ „ „ „
H.21	At the end of oestrus	23·5 „ „ „ „
H.34	15·5 hours before the end of oestrus	24·25 „ „ „ „
H.5	7·75 „ „ „ „	22·0 „ „ „ „
H.9	15·50 „ „ „ „	
	At the end of oestrus	24·0 „ „ „ „
H.13	9·25 hours before the end of oestrus	21·0 „ „ „ „
H.15	9·25 „ „ „ „	21·0 „ „ „ „
H.20	3·0 „ „ „ „	22·5 „ „ „ „
H.6	16·0 „ „ „ „	20·5 „ „ „ „
H.10	8.0 „ „ „ „	17·75 „ „ „ „
H.22	0·25 „ after „ „	22·75 „ „ „ „
H.23	10·0 „ before „ „	24·00 „ „ „ „
H.24	6·0 „ „ „ „	24·25 „ „ „ „
H.25	2·5 „ „ „ „	23·75 „ „ „ „
H.35	15·75 „ „ „ „	24·25 „ „ „ „

Oestrus varied in length from 24 ± 4 hours to 8 ± 4 hours. In two animals, H.9 and H.38, oestrus was interrupted. In H.9 oestrus commenced and lasted for eight hours, ceased for 24 hours, recommenced and lasted for a further eight hours. In H.38 oestrus lasted for eight hours, was interrupted for 16 hours, recommenced and lasted for a further eight hours. H.9 was inseminated 15·5 hours before the end of the first part of oestrus and at the end of the second part. She was examined per rectum 24 hours after the end of the second part and a corpus luteum, estimated to be about 48 hours old, was present in the right ovary. This corpus luteum developed and regressed during the next three weeks, after which the heifer returned to oestrus. H.38 was inseminated 0·25 hours after the end of the first part of heat and 2 hours after the end of the second part. She was examined per rectum 22·75 hours after the end of the second

part when no follicles or corpora lutea were palpable in either ovary and it was assumed that ovulation had occurred. A corpus luteum subsequently developed in the left ovary and when the heifer was killed at 25 days 0·25 hours, a living embryo was present in the left horn of the uterus. Fertilised ova at various stages of development were recovered from all of the six control heifers. The ages and stages of development are shown in Table II.

Table II

Group II Control Heifers

	Time of killing. Hours post-oestrum	*Stage of development of recovered ova*
H.10	40·50	2 cell Resting vesicular nuclei
H.22	65·00	8 „ „ „ „
H.23	55·50	2 „ Division spindles commencing
H.24	40·00	1 „ Metaphase, first cleavage spindle
H.25	64·00	4 „ Resting vesicular nuclei
H.35	55·50	4 „ „ „ „

Twelve of the remaining 18 heifers (including H.38) had not returned to oestrus 24 days after the end of oestrus, and on killing, normal embryos were recovered from nine animals. In the other three no embryo was present in the uterus at *post-mortem* examination, but in two of them (H.2, H.34) foetal membranes were found. A mucous seal was present in the cervix of these three animals and was very large in the case of H.21, in which no membranes were present. A corpus luteum approximating in size to that of pregnancy and of a similar macroscopic appearance was present in the ovary of each of these three animals. The membranes from H.2 disintegrated during fixation and could not be examined microscopically; those from H.24 were serially sectioned. No trace of any embryonic tissue proper could be found, but there was a continuous sac which was free within the uterus, covered on the outside by trophoblast and lined with a continuous layer of endoderm cells. The cells of the trophoblast were enlarged and showed extensive areas of cytolysis; those of the endoderm appeared to be normal (Figs. 1 and 2). The histological sections prepared from the uteri of these three animals were similar in appearance to those from animals about 25 days pregnant. Sections of corpora lutea from H.34 and H.21 were also similar in appearance to those from animals 25 days pregnant. In the corpus luteum from H.2 there were signs of commencing regression indicated by cellular shrinkage and ingrowth of fibrous tissue.

The other six heifers, H.5, H.9, H.13, H.15 and H.20 which returned to oestrus after one cycle, were re-served or re-inseminated. They were subsequently killed and fertilised ova recorded 51·25, 43·75, 71·00, 49·50, 62·50 and 63·50 hours post-oestrum respectively.

Group J

In this group one animal, J.13, did not show normal oestrus at any time during the course of the experiment. At intervals some slight signs appeared but she would never allow service. She was examined per rectum at weekly intervals. The right ovary contained an enlargement thought to be due to a corpus luteum of the size associated with mid-cycle. The condition of the ovaries continued unchanged and at *post-mortem* examination after six months' observation a central cyst 2 cm. in diameter was found. No luteal tissue could be found in this cyst, but patches of old luteal tissue were present in the ovary.

The remaining 23 animals all showed oestrus and were served and inseminated at times between 10.0 hours before and 7.5 hours after the end of oestrus (Table III). They were examined per

TABLE III

INSEMINATION IN RELATION TO OESTRUS (GROUP J)

No.	*Time of insemination*	*Time of examination for ovulation*
J.1	0·5 hours before the end of oestrus	20·5 hours after the end of oestrus
	0·75 " " " "	19·5 " " " "
J.2	10·0 " " " "	20·0 " " " "
	2·25 " " " "	20·25 " " " "
J.3	2·5 " " " "	20·0 " " " "
J.4	2·25 " " " "	20·0 " " " "
J.5	0·25 " " " "	20·0 " " " "
J.6	10·0 " " " "	20·0 " " " "
J.7	At the end of oestrus	20·0 " " " "
J.8	2·25 hours before the end of oestrus	20·0 " " " "
J.9	7·5 " after " "	20·0 " " " "
	6·25 " " " "	19·5 " " " "
J.10	7·0 " " " "	20·0 " " " "
J.11	10·0 " before " "	20·0 " " " "
J.12	0·25 " " " "	19·0 " " " "
J.13	—	—
J.14	0·25 " " " "	20·0 " " " "
	7·5 " after " "	20·0 " " " "
J.15	2·5 " before " "	20·0 " " " "
J.16	9·75 " " " "	20·0 " " " "
J.17	1·75 " " " "	20·0 " " " "
	8·5 " " " "	20·0 " " " "
J.18	6·25 " after " "	19·0 " " " "
J.19	8·5 " before " "	20·0 " " " "
J.20	0·5 " " " "	20·0 " " " "
J.21	10·0 " " " "	19·0 " " " "
J.22	0·5 " " " "	20·0 " " " "
J.23	0·5 " after " "	20.0 " " " "
J.24	2·5 " before " "	20·0 " " " "
	0·5 " " " "	20·0 " " " "

rectum 20 hours after the end of heat to detect whether ovulation had occurred. Seventeen heifers, all of which had ovulated within 20 hours *post-oestrum*, became pregnant at the first oestrus. Six (J.1, J.2, J.9, J.14, J.17 and J.24) returned to oestrus. In the right ovary

of one of these (J.17) a follicle, estimated to be about 2 cm. in diameter, was present 20 hours after the end of oestrus, but at 31 hours *post-oestrum* this follicle was no longer palpable. A corpus luteum subsequently developed in this ovary and remained till the heifer returned to oestrus after a cycle of 20 days. No follicles were palpable in the ovaries of the other five heifers at 20 hours *post-oestrum*. The intervals between the first and second oestrous periods for J.1, J.2, J.9, J.14, J.17 and J.24 were 19, 29, 9, 39, 20 and 39 days respectively.

On re-insemination, four of the heifers (J.1, J.9, J.14 and J.17) became pregnant, and as no Graafian follicles were palpable on rectal examination 20 hours *post-oestrum*, it was concluded that ovulation had occurred. The remaining two (J.2 and J.24) did not become pregnant. J.2 was re-inseminated and at *post-oestrous* examination a follicle of about 1·5 cm. was present in the right ovary. It was still present, but somewhat reduced in size, at 36 hours *post-oestrum* and at 60 hours *post-oestrum* was no longer palpable. A corpus luteum developed subsequently in the right ovary, regressed and after 29 days the heifer returned to oestrus, when she was not served or inseminated. She was killed and at *post-mortem* examination there was no detectable abnormality of the genitalia. J.24 was re-inseminated and had ovulated at 20 hours *post-oestrum*. Five days later what appeared to be a cyst of about 5 cm. in diameter was present in the left ovary. The cyst was ruptured manually and she was not seen in oestrus again during the course of the experiment. She was not pregnant and no abnormality could be detected at *post-mortem* examination at the end of the experiment.

DISCUSSION

It is known that ova die at an early stage after fertilisation in many species. In some, for example, the elephant shrew (*elephantulus myurus jamesoni*), such death is the normal procedure (van der Horst and Gillman, 1941); in others it results in infertile matings. From the evidence of the above experiments, it would appear to occur in cattle.

Oestrus in cattle merges from pro-oestrum into met-oestrum and is of an average length of 13·6 $\pm$ 0·16 hours with a standard deviation of 3·9 hours. The mode is about 14 hours, and 82 per cent. of oestrous periods last between 10 and 18 hours (Asdell, 1946). The signs of oestrus vary very greatly both in their intensity and individual occurrence. They may appear aberrantly at other times of the oestrous cycle (Laing, 1944)* and sometimes during pregnancy (Donald, 1943). Ovulation occurs spontaneously at about 10 to 15 hours *post-oestrum* and slightly earlier in heifers than in cows (Hammond, 1927; Gerasimova, 1938; Brewster *et al.*, 1940; Nalbandov and Casida, 1942). The range in 53 cows studied by Brewster *et al.*

* Unpublished observations.

(1940) was 2 to 26 hours *post-oestrum*. In two out of 72 oestrous periods observed by Nalbandov and Casida (1942) ovulation was delayed for periods varying from 47 to 104 hours *post-oestrum*. The number of spermatozoa required for fertility at natural service is of the order of 1,000 millions per ml. (Laing, 1945b). This number can be reduced at artificial insemination if the semen is deposited beyond the cervix (Salisbury *et al.*, 1943). The quality of the semen must be of a certain minimum level if the spermatozoa are to survive in the female tract until ovulation and then be capable of fertilising (Anderson, 1941 ; Swanson and Herman, 1941 ; Laing, 1945c), and the total survival period of samples of high quality is probably of the order of 30 hours (Trimberger and Davis, 1943 ; Laing, 1945b). The survival time of bovine ova after ovulation is not known, but by analogy with other mammalian ova (Pincus, 1936) and from timed insemination experiments (Trimberger and Davis, 1943) it is probably a few hours only.

In the experiments recorded here an attempt was made to provide all the pre-requisite conditions for fertilisation and to determine whether or not pregnancy followed. It was necessary to control all the variables involved in fertilisation and to know when one or some of them were operating in such a way as to prevent it. The first essential was the accurate determination of the end of oestrus. Mating tests are probably the only reliable criteria because of the extreme variation in intensity of oestrous signs in cattle. By means of such tests the end point was timed to $\pm$ 4 hours and all other events were timed from it. The possibility that spermatozoa might not survive till ovulation was overcome by providing an excess by natural matings and artificial insemination. One natural service always took place within eight hours of the end of oestrus ; the artificial inseminations were all performed within 16 hours before the end of oestrus and in most cases considerably later. The amount and quality of semen provided at artificial insemination was always in itself sufficient for fertilisation provided that ovulation occurred. The remaining known possible variable, delay in ovulation, is more difficult to control and can only be determined by rectal examination at some time *post-oestrum*. The main difficulty lies in deciding when the examination should be made. If 13 hours *post-oestrum* is considered as the average time of ovulation, examinations made too near to this point involve the danger of damaging a normally maturing follicle and causing either luteinisation without ovulation, or rupture of the follicle into the peritoneal cavity. Examinations made earlier than 13 hours *post-oestrum* entail the possibility of including animals which ovulate after the spermatozoa have either died or ceased to be capable of fertilising. In the case of Group H, 24 hours *post-oestrum* was selected arbitrarily as a possible safe mean between these two risks ; in Group J the time was reduced to 20 hours to provide stricter control. The recovery of fertilised ova from all the controls of Group H and also from the six heifers in this group

which returned to oestrus and were re-inseminated before being killed, confirms that the conditions of the experiment were such that fertilisation always took place, or that its absence could be detected.

The evidence of Group H suggests that ova died after fertilisation and that the mortality was of two forms. In the first, the ovum was fertilised, developed for some days but died before mid-cycle, the corpus luteum regressing as in a normal cycle where fertilisation has not occurred and the animal returning to oestrus after one normal oestrus period without any evidence that fertilisation had occurred (H.5, H.6, H.13, H.15, H.20). In the second, fertilisation occurred and the ovum developed beyond mid-cycle so that the corpus luteum persisted; the embryo then commenced to degenerate and be resorbed, and the corpus luteum regressed but after a length of time greater than one oestrous cycle (H.2, H.21, H.34). The evidence for the occurrence of the first is indirect, as owing to the conditions of the experiment no degenerate ova were recovered, but it is not so indirect as to be inconclusive. Abnormal ova had also been recovered previously from other animals (Figs. 3 and 4). Direct evidence was obtained of the occurrence of the second type from the heifers H.2, H.21 and H.34.

From Group J confirmatory evidence was obtained of the occurrence of both these varieties of the condition. Three animals returned to oestrus after a length of time equal to or less than one normal oestrous cycle (J.3, 19 days; J.5, 9 days; J.24, 20 days), and two after a time greater than one normal cycle (J.4, 29 days; J.6, 39 days).

A point which is of some importance with regard to the influence of the condition on the general incidence of infertility is whether it occurs at successive periods in the same animal. The data from this point of view is rather scanty, but one animal (J.24) was infertile, presumably following death of a fertilised ovum, at two successive cycles, but the picture was complicated by the occurrence of an ovarian cyst five days after the second period.

The difference in incidence of the condition in Group H and J is also of interest. In Group H, eight of 18 animals were affected, or for purposes of comparison with Group J, 44·4 per cent, three heifers (16·7 per cent.) not returning to oestrus after one cycle and five (27·7 per cent.) returning after one cycle. Group J had a total incidence at the first oestrous periods of five heifers out of 24 (20·8 per cent.), two (8·3 per cent.) of the first type, and three (12·5 per cent.) of the second type. This may be explained from the fact that Group H was composed to some extent of animals culled from dairy herds and that culling was due in most cases to failure to breed, while Group J was, from the reproductive aspect, rather better than a random sample of commercial heifers. It is possible that the incidence of embryonic mortality under the conditions of experiment gives some indication of the incidence amongst commercial cattle under similar conditions. It might be fair to suggest that such

incidence would fall between that of the two groups, perhaps at about 30 per cent.

Although these experiments seem to establish that embryonic death occurs at an early stage in apparently normal cattle and may play an important part in causing infertility, they shed little light on its aetiology. Histological examination of the genitalia was negative. The experimental animals were maiden heifers mated to disease-free bulls and there was no evidence of bacterial or protozoal infection. Nevertheless, embryonic death at an early stage in the absence of infection is explainable on the basis of the occurrence of one or more, or a combination of three conditions : (1) The ova are inherently or through some acquired defect incapable of continuous development ; (2) the spermatozoon concerned in fertilisation does not provide a pro-nucleus able to play its part in normal growth ; (3) through failure in progesterone production, intra-uterine conditions are such that a fertilised ovum cannot be maintained.

The evidence of Group H is against the last of these three being responsible, if it can be taken that it would result in detectable histological changes in the structure of the endometrium and perhaps also of the corpus luteum. No indication of such changes was found in H.2, H.21 and H.34.

Defects in spermatozoa may occur at any time before ejaculation. To postulate inherent defects would involve these being present in spermatozoa from several different bulls at varying times and would require the presence of such defects in a high proportion of the sperm-population produced by them, when the relatively high incidence of the embryonic mortality is considered. Acquisition of defects by spermatozoan may also be associated with ageing in the female tract. By the conditions of the experiment some spermatozoa must have been present in the tract for times exceeding their optimum, but the numbers of healthy spermatozoa must always have been greater and it seems reasonable to assume that fertilisation by one of these latter was more likely.

Pathological changes in ova can also occur before or after ovulation and in the latter case are usually associated with some delay having occurred before fertilisation took place. In these experiments, spermatozoa must always have been present in the Fallopian tube when ovulation occurred and the latter possibility can probably be excluded. Support for the view that pathological changes were occurring in the ova before ovulation is given by the data from heifers which were infertile from causes other than embryonic death. All these cases were associated with some anomaly of the oestrous cycle—interrupted oestrus in H.9, delayed ovulation in J.2 and J.17, and formation of an ovarian cyst in J.24. Heifer J.13 can be excluded from consideration as her condition was established before the start of the experiment and remained unchanged throughout its course and until slaughter some months later. The evidence from these animals and from the others in which embryonic mortality

occurred, suggests that under the particular conditions of experiment follicle formation was abnormal. This was expressed in different animals by interrupted oestrus, delayed ovulation, production of ova which could be fertilised but could not develop, or in formation of an ovarian cyst.

Infertility in apparently normal cattle occurs at all seasons of the year, but the incidence is highest during the winter months from October to March. In the most extreme form, which occurs most commonly in heifers during the winter, the ovaries are completely quiescent, no follicles develop and no oestrous cycles or periods occur. In autumn-calved cows, lactating during the winter, oestrous cycles are often irregular and oestrous periods short and ill-defined. Among those animals which are mated, the conception rate may be low. Very often, although corpora lutea develop cyclically in the ovaries, oestrus is never observed. It is not known definitely whether such cycles are abnormal only in the briefness and indefiniteness of the oestrous periods, or whether in some cases oestrus and ovulation do not occur, although the follicle luteinises. Delay in ovulation is sometimes said to take place, although the diagnosis in the field is made somewhat uncertain because of the absence of any reliable criterion of the end of oestrus. Although this syndrome is most common in lactating cows, and complete anoestrum most frequent in heifers, anoestrum does occur in cows and the less extreme condition in heifers. The incidence of both conditions appears to be higher in the more northerly counties and in severe winters. They occur in animals at all levels of nutrition, but seem more common when it is quantitatively deficient. In summer so-called "featureless" infertility is uncommon and sometimes characterised by irregular oestrous cycles. Oestrus is usually of normal length.

The main external factors involved in reproduction, *viz.*, light, nutrition and temperature, have been reviewed by Marshall (1936, 1941). Under natural conditions each species reacts differently to exteroceptive factors so as to have a definite breeding season. Domestic cattle will breed throughout the year if their environment is suitably supplemented, but where this is not done, as a result of the condition outlined above, they tend to revert to spring-calving as spontaneous resolution occurs and the animals become fertile in the spring and early summer when they go out to grass.

The conditions of the foregoing experiments do not correspond exactly to any system of husbandry. Although the heifers were kept in yards and fed hay and concentrates, they were living under conditions of light and temperature exposure which were similar to those for animals at grass. In effect, the conditions approximated to an average between normal winter and summer husbandry of breeding stock. The fertility obtained was also an approximation between the low level of winter breeding and the high fertility which usually obtains in summer. All gradations of fertility must exist between complete anoestrum and normal follicle maturation with full observ-

able oesterus and ovulation at the proper time. The various degrees of anoestrum, delayed ovulation, and early embryonic death are probably transitional stages. In the above-described experiments the phase associated with embryonic mortality was most marked, but if the deficiencies of the experimental animals' environment had been varied other phases might have predominated.

The conditions of experiment were not sufficiently precise to give any indication of the basic aetiology of the infertility. From previous work (Marshall, 1936, 1941) multiple factors appear to be involved. It would be of interest to determine the effects of varying combinations of the environmental factors in producing and preventing embryonic mortality and related conditions. Experimental variation of hormone levels at oestrus might also give some indication of the immediate aetiology of infertility of these types.

SUMMARY

Forty-eight heifers, in two groups of 24, were mated, in successive years, under controlled conditions to determine whether infertility resulted from death of fertilised ova.

The condition was found to occur in both groups.

The aetiology of the infertility is discussed and it is suggested that it may be associated with anomalies of the later stages of follicle maturation.

The relationship of the condition to general bovine infertility is considered.

ACKNOWLEDGMENTS

This work was carried out under the direction of Professor T. Dalling, to whom I am indebted for much help and encouragement. I must thank also Dr. J. Hammond, F.R.S., for much advice and help and for accommodation in his department during the course of the work, Mr. W. S. Mansfield, for providing yards in which to keep the experimental animals, and Mr. J. A. Macmillan for providing some of the heifers.

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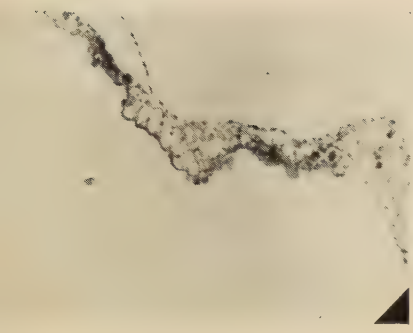

FIG. 1.—Vacuolation of trophoblast. × 60, H. and E.

FIG. 2. — Normal trophoblast. × 60, H. and E.

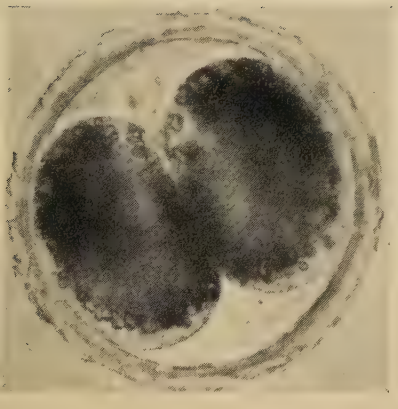

FIG. 3.—Two cell ovum, 45 hours *post-oestrum* showing peripheral cytolysis. × 230 unstained.

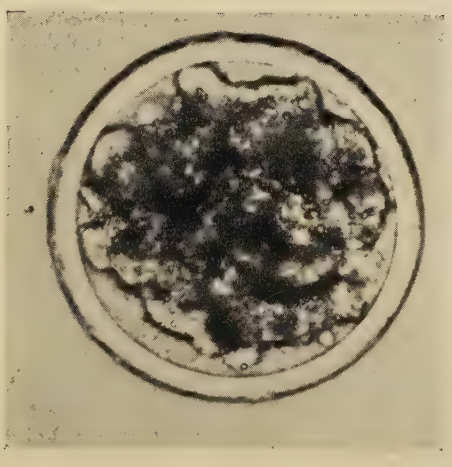

FIG. 4.—Early morula, 72 hours *post-oestrum* showing generalised degeneration. × 230 unstained.

THE PRODUCTION OF HYALURONIDASE BY *STREPTOCOCCUS AGALACTIAE* (LANCEFIELD'S GROUP B)

By

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INTRODUCTION

Hyaluronidase has been derived from many sources including the mammalian testicle, leeches, snake and spider venoms, malignant tumours and from a wide variety of pathogenic organisms including streptococci. Duran-Reynals (1933) considered that hyaluronidase secretion was characteristic of invasive strains of streptococci and McClean (1941) found, in the case of Group A and C streptococci, that the enzyme was produced only by non-capsulated strains. This observation was confirmed by Crowley (1944). Hyaluronidase production by Group B streptococci has not been extensively studied. A few strains were examined by Stewart (1938) and by McClean (1941) and, with one exception, the enzyme was demonstrated. In view of the importance of *Str. agalactiae* in the causation of mastitis and our lack of knowledge concerning its mechanism of infection and spread, it was considered desirable to make a more extensive study of the production of hyaluronidase by this organism.

METHODS

Most of the strains examined were isolated either from clinical or subclinical cases of bovine mastitis or from the skin of cows. A few strains of known serological types, obtained from the N.C.T.C. and from the Ministry of Agriculture Research Laboratories, were also tested.

Two tests were used for the detection of hyaluronidase. In the first instance the culture supernatants of a few strains were tested for their power to diffuse through the rabbit's dermis, using the technique described by McClean and Hale (1940). This method was later replaced by the mucin clot prevention test, (McClean *et al.*, 1943), which depends on the fact that hyaluronidases destroy the property of a mixture of hyaluronic acid and protein to form a mucin clot. The test substrate consisted of a mixture of equal parts of a 0·2 to 0·25 per cent. solution of potassium hyaluronate and horse serum diluted twice in distilled water. Suitable dilutions of culture supernatant were added in 0·5 ml. amounts to 1 ml. of the substrate, and after incubation for 20 minutes at 37° C. followed by immediate cooling, 0·2 ml. of 2N acetic acid was added and the presence or absence of a mucin clot noted. The potassium salt of hyaluronic acid, a substance present in skin, synovial fluid, vitreous humour and connective tissue, was prepared from human umbilical cords. At the commencement of this study the possibility of pre-

paring the substance from cords of domestic species was explored with disappointing results. No hyaluronate was obtained from the umbilical cords of the mare and the cow and those of the ewe gave a yield of approximately one tenth that obtained from a similar weight of human cords.

RESULTS

Using the skin diffusion test hyaluronidase production was demonstrated in seven strains of *Str. agalactiae,* isolated from cases of mastitis. A laboratory strain, G104, did not produce a significant degree of diffusion although, after mouse passage, its titre increased tenfold. No increase in the diffusing powers of other strains subjected to mouse passage could be demonstrated. This test gave qualitative rather than quantitative results and was not suitable for determining the relative amounts of enzyme produced by various strains. The mucin clot prevention test was therefore used for comparison of strains and the following table summarises the results with type cultures and cultures isolated from cases of mastitis and from the skin of the udder.

HYALURONIDASE PRODUCTION BY *Streptococcus Agalactiae*

Serologically typed strains		*Clinical mastitis strains*		*Subclinical mastitis strains*		*Skin strains*	
Strain	*Titre*	*Strain*	*Titre*	*Strain*	*Titre*	*Strain*	*Titre*
S119 (1a)	1 : 10	7L	1 : 20	7L	1 : 80	7S	1 : 80
S127 (1b)	1 : 10	W41	1 : 40	5L	1 : 20	AS	1 : 40
G19 (1c)	1 : 10	E	1 : 20	W3	1 : 80	58	1 : 40
G2 (1a)	1 : 10	LRF	1 : 40	6L	1 : 80	WS	1 : 80
S121 (2a)	1 : 100	C	1 : 20	WR	1 : 80		
S101 (3a)	1 : 100	La	1 : 80	8L	1 : 20		
S106 (3b)	1 : 10	3	1 : 20	Lady	1 : 40		
G104 (3c)	Trace	L	1 : 40	M	1 : 20		
		HS	1 : 80	9R	1 : 40		
		FB	1 : 40	HCS	1 : 20		
		KW25	1 : 40	SL68	1 : 40		
		LS	1 : 80	SL27	1 : 40		
		SG	1 : 80	SL46	1 : 20		
		KW6	1 : 80	SL73	1 : 80		
		ARC3	1 : 40				
		DL	1 : 20				
		H16	1 : 80				
		W37	1 : 40				
		K2	1 : 20				

In the examination of the type cultures a tenfold dilution series of the culture supernatant was employed but, although all produced hyaluronidase, a better end-point was obtained using two fold dilutions. In all strains tested the enzyme titre seldom exceeded 1 : 80 and no relation was found to exist between the source of the strain and the amount of hyaluronidase it secreted. A few variant forms

were tested with similar results and it is appropriate to note at this point that Meyer *et al.* (1941) recorded similar findings in an examination of *Str. pneumoniae* variants. Serial passage of *Str. agalactiae* through mice did not increase hyaluronidase activity although in some cases virulence was enhanced, but a slight increase in activity was obtained by including potassium hyaluronate in the medium. The following medium was found suitable for routine examinations: horse meat infusion broth containing 2·5 per cent. Evan's peptone and 0·25 per cent. potassium hyaluronate.

McClean's observation that hyaluronidase production was common to both capsulated and non-capsulated strains of *Str. agalactiae* has been confirmed. It was possible to demonstrate capsules by negative staining methods in the majority of field strains and, although capsules were sometimes lost after prolonged subculture, there was no diminution in hyaluronidase production.

DISCUSSION

Hyaluronidase or diffusion factor was produced by 52 strains of *Str. agalactiae* of bovine origin. Their ability to secrete this enzyme was of a low order: moreover, with one exception, it was not enhanced by mouse passage and was only slightly increased by including the specific substrate, hyaluronic acid, in the growth medium. These findings were in contrast to the effect of such treatments on *Cl. welchii* and non-capsulated Group A streptococci when a dramatic increase in enzyme activity occurs. It must be noted, however, that whereas these organisms were of a relatively high virulence, the Group B strains examined were non-pathogenic.

Although the strains differed in their hyaluronidase productivity the variation was very slight and bore no relation to the clinical severity of the disease from whence the strain was derived. Similar findings have been recorded in the case of Group A streptococci isolated from human infections (Crowley, 1944) and in *Str. pneumoniae* isolated from cases of pneumonia (Humphrey, 1944).

The role of bacterial hyaluronidase in infection is not clearly understood although it is possible that the enzyme, by increasing the permeability of the tissues, accelerates the dissemination of the bacteria and their products. Such a hypothesis may explain certain features of the clinical picture seen in human erysipelas and in gas gangrene, but cannot be said to hold true in the case of a localised infection such as bovine mastitis associated with *Str. agalactiae*. In this disease, although hyaluronidase may be considered as one of the many factors influencing the spread of infection within the mammary tissues, the result of this investigation, where hyaluronidase production by strains of *Str. agalactiae* has been shown to be uniformly low, does not suggest that the enzyme plays a decisive role in the infective process.

SUMMARY

Fifty-two strains of *Str. agalactiae* have been tested for hyaluronidase production. All showed hyaluronidase activity of a low order but no relation could be demonstrated between the source of a strain and its activity.

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PLASMA-CELL MYELOMATOSIS IN THE DOG

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INTRODUCTION

Multiple myelomatosis is somewhat uncommon in man, representing about 0·03 per cent. of all types of malignancy (Geschickter and Copeland, 1928). It is a disease of middle and late life and is said to be twice as common in the male as in the female. The literature on the condition in man is extensive but there is apparently no reference to the condition in the dog. It is thought, therefore, that this is probably the first recorded instance of a plasmocytoma of canine origin.

Geschickter and Copeland (1936) described the condition in man in detail and gave numerous references. The tumour is said to arise in the marrow tissue; there is multiple involvement of the skeleton and, in some cases, the viscera. Salient clinical features include pain, deformities and fractures of bone, multiplicity of lesions, nephrosis and excretion of Bence-Jones protein in the urine. Although the greater number of cases show the lesions confined to the marrow, Willis (1948) stated that in a proportion of fatal cases "Localised tumours or diffuse infiltrations of the characteristic cells are present in the viscera or other soft tissues." Churg and Gordon (1942) considered visceral involvement rare and described a case with extensive involvement of the spleen and, to a lesser extent, the liver and lymph nodes. Wintrobe (1946) listed the viscera which have been involved in myelomatosis, as follows: liver, spleen, lymph-nodes, kidneys, ovaries, testes, adrenals, heart, skin, subcutaneous tissue, tonsils, thyroid, pleura, gastro-intestinal tract and uterus. A few cases of primary plasmocytomas in soft tissues have been described (Hellwig, 1943; Claiborn and Ferris, 1931; Schwander, Estes and Cooper, 1948). The most frequent sites of origin for these primary tumours are said to be the nasal cavity and the nasopharynx, but other sites are described. In a number of cases amyloidosis has been associated with these extensive lesions (Wintrobe, 1946). Fodden (1947) described a case of systemic amyloidosis associated with a plasma cell myeloma in the sternum and pelvis.

The predominant cell in these neoplasms is the plasma cell or its precursors or derivatives. Marked variations in the morphology of the main cell present have been noted by all workers, but the majority of the tumours are of the plasma cell type. A great many of the neoplasms are composed of atypical plasma cells and the literature refers to many types of myeloma, *e.g.*, myeloblastic, lymphoblastic, haemocytoblastic, myelocytic and lymphocytic. In some cases a single lesion contains many different types of cells; for example, Smith and Silverberg (1936) described a myeloma, the cellular elements of which were said to be a mixture of lymphocytes, plasma cells, immature red cells, megakaryocytes and haemocyto-

blasts. The myeloma cell is described by Wintrobe (1946) as "moderately large (15μ to 30μ) round or ovoid and contains a nucleus about 5μ to 7μ in diameter. The latter is round, eccentrically placed, and contains one, sometimes two, nucleoli."

CASE-HISTORY

Subject.—Crossbred terrier, male; aged eight years.

When first seen the dog had a large hard swelling in the submaxillary region, and had pyrexia. The swelling appeared to soften and was opened surgically. There was some escape of a thick fluid from the lesion. The temperature remained high and the swelling increased in size. A biopsy was performed.

The tissue consisted of a large soft mass, pinkish grey in colour and semi-liquid in parts. Bacteriological examinations gave negative results. A study of smears revealed very large numbers of cells despite the fact that the slides had been very lightly pressed on to the tissue. The majority of the cells appeared to be plasma cells, but erythrocytes, lymphocytes, myelocytes and a few neutrophils were seen, in addition to cells, the nature of which was not apparent. The latter were about the size of lymphoblasts, but many of them had basophilic granules in the cytoplasm. A few isolated normoblasts were seen. The plasma cells were fairly typical with their slightly basophilic cytoplasm and eccentric nucleus. In many cases the "cartwheel" appearance of the nuclear chromatin was very noticeable. One or two in each field showed two nuclei. These cells did not stain with an Unna Pappenheim stain.

The most prominent feature of the sections was the cellularity of the tissue. The bulk of the neoplasm consisted of masses of free cells with a very scanty stroma. There were few collagen fibres and little reticulum. The tissue was well vascularised and there were several areas in which there had been haemorrhage. The blood vessels were primitive and consisted of little other than a cavity lined by endothelium. Many free erythrocytes were present among the tumour cells which varied in size and appearance, but the predominant cell was the plasma cell. The cytology in general followed that seen in the stained smears; in the main there was no intercellular stroma and such connective tissue as was present was distributed around the blood vessels. The density of the plasma cells varied from field to field; in some areas the cells formed dense masses whereas in others they were in a loose arrangement and apparently unsupported. Mitotic figures were numerous and all the nuclei were hyperchromic.

At this stage a tentative diagnosis of plasmocytoma was made. The dog was in poor condition with a staring coat and pale mucosae. The intermaxillary space was completely filled with new growth (there had been rapid growth following the biopsy) and this was infiltrating into the neck region. The mass of tissue was firm to the

touch and insensitive. The animal's temperature was normal. The growth was so diffuse that surgical interference was out of the question and the animal was destroyed.

Blood Examinations.—The following figures were obtained from an examination of the blood: Red blood corpuscles, 4·0 millions per c.mm.; white blood corpuscles, 20 thousand per c.mm.; haemoglobin, 9·8 G per cent. A differential count indicated: Neutrophils, 60 per cent.; lymphocytes, 30 per cent.; monocytes, 5 per cent.; eosinophils, 4 per cent.; basophils, 1 per cent. Although the sedimentation rate was not determined, it was noted that the corpuscles settled very rapidly.

Urine Examinations.—There was a trace of albumin and examination of the centrifuged deposit showed the presence of a few granular casts, transitional epithelial cells and very few erythrocytes. Careful examination for Bence-Jones protein gave indefinite results.

Post-mortem Examination.—The lesion in the intermaxillary space was diffuse and measured approximately 6 in. × 6½ in. × 2 in. The neoplasm had infiltrated into all the tissues in this area and had spread extensively in the pharyngeal region. The larynx was almost completely covered and only the epiglottis could be distinguished. The submaxillary and superior cervical lymph nodes formed part of this mass. The tissue was soft and greyish-pink, while many areas appeared to be degenerated and were semi-gelatinous. Haemorrhagic areas were also noted.

The thoracic viscera were macroscopically normal. The liver was enlarged, very friable and had a mottled appearance due to innumerable greyish areas scattered throughout the liver substance. The spleen was enlarged and had a similar mottled appearance. The gastro-intestinal tract was normal to the naked eye. The kidneys were small, somewhat granular with an adherent capsule, and on section revealed grey infarcts in the cortex of both organs. A femur and humerus were split longitudinally and fairly well defined greyish areas were noted; the remainder of the marrow showed red oedematous changes. A radiological examination of remaining bones, including the skull, did not show any obvious abnormality; there did not appear to be any rarefaction, and no typical punched-out areas of a myeloma were noted. The marrow, however, did show dark areas, which were found to be identical with those noted in the femur and humerus.

Histological Examinations

Liver.—The hepatic tissue was diffusely and extensively infiltrated with cells. There was marked loss of liver tissue and the cells which remained were widely separated by oedema, plasma cells and extravasated blood (Fig. 2). The liver cells showed all stages of degeneration from cloudy swelling to actual necrosis. It appeared as if the infiltration had begun in the area of the intralobular vein and had then spread peripherally. Large areas of liver had disappeared and been replaced by collections of loose

plasma cells and lymphocytes. As in the tissue from the neck the plasma cell areas had little stroma. The cells were, in the main, of the plasma type but there appeared to be more variation in the size and shape than was noted in the neck lesion. Nucleoli were prominent in some cells and others had a well defined nuclear membrane.

Spleen.—There was almost complete loss of splenic structure. With low magnification the spleen appeared to be a mass of loose cells with a few isolated trabeculae : very few lymph follicles remained. The spleen tissue was extensively infiltrated with myeloma cells. Lymphocytes and lymphoblasts were also present in large numbers (Fig. 3).

Kidneys.—There was some general fibrosis, most marked around the vessels and around Bowman's capsules. A few tubules were dilated and two small cysts were seen in one section. The vessels were congested, there had been some haemorrhage and erythrocytes were lying in the lumen of some of the tubules many of which were severely damaged and showed necrosis and desquamation of the lining epithelium. The straight tubules were severely damaged. Many glomeruli were tufted and well nucleated, a few isolated ones were fibrosed. Hyaline casts were present in some tubules and there was extensive deposition of amyloid material in and around the convoluted tubules (Fig. 4).

Bone-marrow.—The marrow was examined by means of smears and in section. The main feature was the degree of cellularity and the absence of fat. Masses of plasma cells were found and as a general rule they tended to be grouped together although there was some infiltration. Relatively few normoblasts were present, and although there were many large primitive, undifferentiated granular leucocytes, few myelocytes were to be seen. A few large plasma cells were present and there appeared to be all gradations from this cell to large lymphoblasts and myeloblasts.

DISCUSSION

The histogenesis of this tumour is of importance since it may possibly throw light on the origin of the plasma cell. As yet, the origin of this cell has not been determined and much of the work done on the myeloma has been done with this end in view. The cytology of many of the neoplasms described as myelomas varies a good deal and attempts have been made to classify them according to the predominant cell present. Willis (1948) stated that in two of his cases the cells may have been " any kind of immature haemopoietic cell." He, together with Geschickter and Copeland, considered that in these uncertain cases the cells should be regarded as myeloma cells and stated that they were probably immature forms of variants of the typical plasma cell. The customary view of the plasma cell is that it is related to the lymphocyte. This is not out of place with many of the suggestions regarding the origin of the cell in the plasmocytoma. Geschickter and Copeland (1936) thought that the cell might arise from an " as yet unrecognised specific marrow cell." Smith and Silverberg (1936) concluded that the myeloma arose from a primitive cell, the haemocytoblast, which they said was the specific marrow cell of Geschickter and Copeland, and was said to retain its proliferative capacities throughout life. Churg

and Gordon (1942) thought it originated from a multipotential haemic cell in the reticulum of bone marrow. The observation of Cappell (1929) on the occurrence of transitional forms between plasma cells and myeloblasts and possible transitional forms between myeloma cells and fibre-producing cells also suggests that the myeloma plasma cells are variants of undifferentiated reticulum cells. Ewing (1942) summarised the views of various workers on the possible origin of the cells : they include adventitial cells of blood vessels, lymphoblasts, myeloblasts, erythroblasts and a common stem cell which has many stages of differentiation. Lowenhaupt (1945) defined the tumour as " a local tumour of reticulo-endothelial origin, with or without multiple metastases." He suggested that the cells arose from histiocytes and thought the plasma cells were specifically related to the cells concerned in antibody production.

In the case presented here the gross and microscopical appearances resembled closely the condition described in man. An unusual feature was the difference in the skeletal lesion. Although the marrow tumours were numerous there was no bone resorption and X-ray examination failed to show the characteristic punched-out appearance of bone described in human cases. This may have been due to the fact that the tumours were not sufficiently developed or it may be due to a species difference. The distribution of the hepatic and splenic lesions was leukaemoid in type rather than metastatic. These organs may be regarded as parts of the haemopoietic system and multiplication of either an undifferentiated haemic cell or a cell of reticulo-endothelial origin would explain the distribution of these lesions in a more satisfactory manner than would metastatic spread. The lesions did not appear to be embolic in origin. The pleomorphism of the cells also suggested a multifocal origin. It is, of course, possible that metastatic spread may occur in some cases (as it undoubtedly does in some leukaemias), and this would explain the presence of lesions in non-haemopoietic tissues such as kidney in some of the cases described.

The size, and especially the history of rapid growth of the lesion in the intermaxillary space, is of interest, but it is doubtful if this was the primary lesion. Its origin may have been in the submaxillary lymph nodes, or in the nasopharynx. There was no involvement of the underlying maxillae. The kidney lesions resembled those seen in human cases, especially in respect of the extensive tubular damage. No great importance was attached to the fibrotic changes since chronic nephritis is very common in old dogs and is frequently encountered at *post-mortem* examinations. The urinary findings were not of significance since they, too, are associated with chronic nephritis. The indefinite result obtained in the test for Bence-Jones protein is not surprising since many authors are in agreement that the presence of this protein in some cases of multiple myeloma is intermittent and it is not generally present constantly until the late stages of the disease.

SUMMARY

A case of multiple myeloma (plasmocytoma) in a dog is described. The lesions resembled those seen in man. It is suggested that the neoplasm was multifocal in origin and probably arose from the reticulo-endothelial system.

ACKNOWLEDGMENTS

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Fig. 1

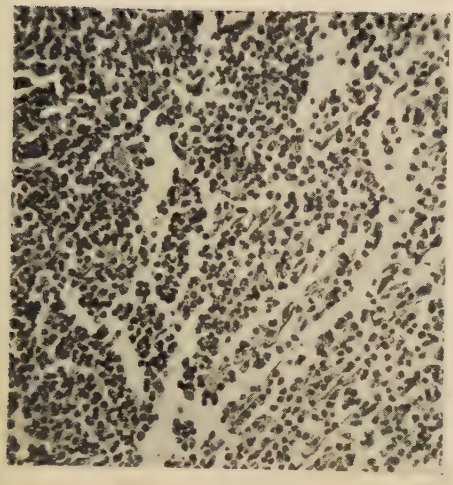
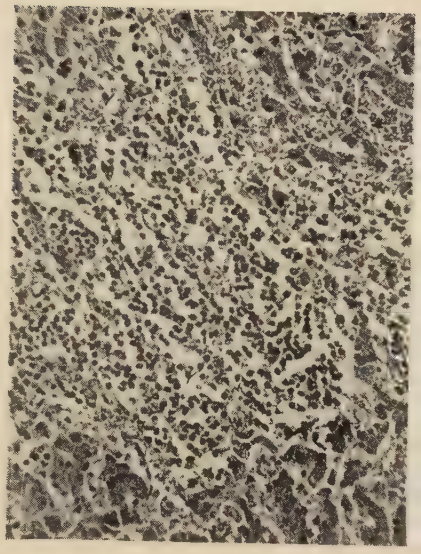


Fig. 2



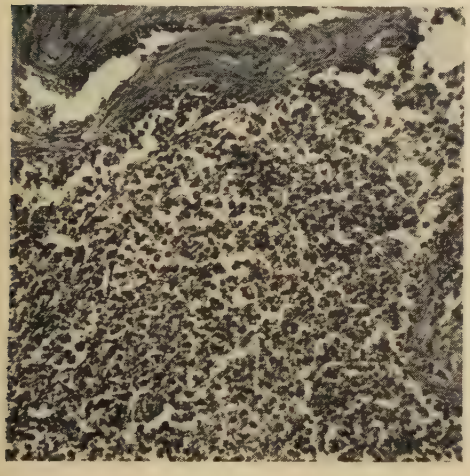


Fig. 3

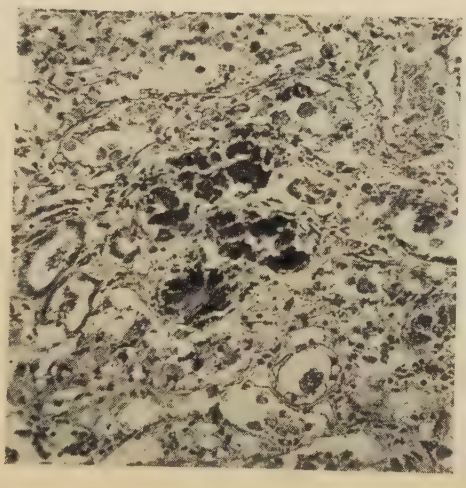


Fig. 4

FIG. 1.—Local lesion, showing loose cellular structure and scanty stroma. Haematoxylin and eosin. × 100.

FIG. 2.—Liver. Infiltration of liver and loss of liver substance. Haematoxylin and eosin. × 100.

FIG. 3.—Spleen showing loss of splenic architecture and replacement by plasma cells. Haematoxylin and eosin. × 100.

FIG. 4.—Kidney showing amyloid deposits and degenerative changes in the tubules. Haematoxylin and eosin. × 100.

INFECTIOUS ENTEROHEPATITIS (BLACKHEAD) IN TURKEYS: A COMPARATIVE STUDY OF METHODS OF INFECTION

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INTRODUCTION

In the course of investigations into the prevention and treatment of blackhead, one of the major difficulties has always been the lack of an adequate supply of infected turkeys under controlled conditions. This is well illustrated by the recent work of Temperton, *et al.* (1947), Temperton *et al.* (1948), in which it was not found possible to estimate the prophylactic value of neoarsphenamine and other drugs. These workers relied on natural infection and the incidence of disease in the different treated groups, including the controls, was not significant. For statistical purposes it is essential that diseased birds should be of comparable age and weight in order that the efficiency of preventive or curative methods can be accurately estimated. Furthermore, it is desirable that as far as possible the incidence of blackhead in groups of birds used for experimental purposes should attain 100 per cent. It is therefore important to have a method of producing blackhead at will. With this object in view a number of experiments were made and these form the subject of this communication.

At the outset it was necessary to review the work of others in this field and an attempt has been made to summarise the literature, with particular reference to artificial infection. Since the original work by Theobald Smith (1895), many attempts have been made to reproduce the disease. Various methods have been used and, in our opinion, the following appeared most promising for further investigations: (1) Infection by cultures; (2) rectal and oral administration of infective material; (3) feeding embryonated heterakis eggs; and (4) exposure to contaminated ground. The results obtained by other workers are as follows:

Infection by Means of Cultures of Protozoa.—DeVolt and Davis (1936) obtained 87·9 per cent. infection by inoculating birds with cultures of *Histomonas meleagridis* at repeated intervals. DeVolt and Holst (1948) used a similar technique and infected 27 out of the 28 poults inoculated (96·4 per cent.). Rectal inoculation with young cultures of the same organism was attempted by Tyzzer (1936) who obtained 99 per cent. infection in 161 out of 162 hen chicks, but those subcultured for lengthy periods became attenuated and did not produce blackhead. Tyzzer (1934) infected by the rectal route 15 out of 16 poults and 18 out of 25 hen chicks. Allen (1941) fed turkeys with cultures of *Trichomonas gallinarum* and *Histomonas meleagridis* and produced trichomoniasis in 13·3 per cent. and histomoniasis in 92·3 per

cent. Bishop (1938) infected hen chicks by rectal inoculation of cultures of *Histomonas meleagridis*.

Infection by Administration of Caecal and Liver Lesions.—Tyzzer and Collier (1925) succeeded in producing blackhead in 38 per cent., *i.e.*, seven out of 18, of the birds used. Tyzzer (1932), using only four poults, infected two of them. Wegeforth and Wegeforth (1921) infected four out of six poults (66 per cent.), but Graybill and Smith (1920) were unable to obtain positive results with this technique. Rectal infection was used by Tyzzer and Collier (1925) who achieved 80 per cent. infection using 20 poults, and Tyzzer (1933) who obtained 100 per cent. infection in ten hen chicks. Delaplane (1932) reported 84 per cent. infection with turkeys and 62 per cent. with hen chicks.

Running Turkeys on Infected Ground.—Graybill (1921) produced 12 per cent. infection in a flock of artificially-reared turkeys. Smith (1917) obtained 48 per cent. infection in young poults exposed to infected ground along with yearling turkey hens. Rettger and Kirkpatrick (1927), and Rettger, Kirkpatrick and McAlpine (1929) reported an incidence of 17 to 21 per cent. for birds kept in stationary pens as against 7 to 8 per cent. for birds on a rotation system. DeVolt and Davis (1936) gave an incidence of 46·2 per cent. in poults and 15 per cent. in adult turkeys.

Running Turkeys with Fowls.—Smith (1917) mixed turkeys with yearling hens but no deaths resulted. Tyzzer, Fabyan and Foot (1921) ran common fowls with one turkey which subsequently developed blackhead. DeVolt and Davis (1936) kept poults with chicks and produced blackhead in 62·5 per cent. of the birds exposed.

Although it will be seen from the above summary that a considerable amount of work has been done on the problem of blackhead infection, it has, on the whole, fallen short of what might have been achieved had more attention been paid to the number of birds used and to the use of control birds at the same time. This is well illustrated by the results of Allen (1941) in which a high infection rate was produced by means of cultures, but an incidence of 42 per cent. infection in the controls invalidates the result. Furthermore, all birds under test were kept under field conditions and infection did not develop until three to seven months after inoculation. There is no evidence to prove that infection did not, in fact, take place from the ground alone and the lengthy incubation period before infection was apparent suggests that the cultures were not responsible for the disease.

EXPERIMENTAL WORK ON BLACKHEAD INFECTION

Three sets of experiments were carried out with the following objects in view: (1) To find the incidence of blackhead produced by running turkey poults on infected ground; (2) to infect turkeys by rectal injection of cultures of *Histomonas meleagridis*; (3) to infect turkeys by the inoculation of diseased tissue.

Infection by Contact with Infected Ground

Eleven groups of turkey poults, six to eight weeks old, were run

on infected ground, *i.e.*, this ground had previously accommodated turkeys which had developed blackhead. The numbers in each group varied with the hatch and are given along with other details in Table I. *Post-mortem* examinations were made on all birds dying during the course of the experiment, while those birds showing clinical symptoms were also killed and examined. Diagnosis of blackhead depended on the presence of typical liver and caecal lesions.

TABLE I

INFECTION BY CONTACT WITH INFECTED GROUND

Group	*No. of turkeys in group*	*No. of deaths from blackhead*	*Deaths from other causes*	*Length of time on infected ground*
A2	25	5	1	15 weeks
A1	19	2	3	20 ,,
A3	30	0	2	10 ,,
B2	21	5	1	13 ,,
B3	19	0	1	10 ,,
C1	28	5	1	18 ,,
C2	30	0	1	10 ,,
D2	34	0	0	10 ,,
E	37	28	0	8 ,,
D1	15	1	2	18 ,,
B1	30	3	4	20 ,,

Out of a total of 288 birds run on infected ground, 49 developed blackhead representing 13·4 per cent. of the total number of birds. A very high incidence of infection was noted in Group E, namely, 28 out of 37 or 75 per cent. It was found that poultry had previously been kept on this ground for some time, and in the light of this result another experiment was carried out to determine the influence of poultry in inducing the disease.

Sixty-nine turkeys, six to eight weeks old, were divided into three equal groups. The first group was run on ground with 18 hens, the second group was run on infected ground with no hens, *i.e.*, formerly used by Group E, and the third group was run on " clean " ground, *i.e.*, not previously occupied by infected turkeys or hens. These conditions were maintained for one month ; all birds dying during the experiment were examined *post-mortem* and at the end of the experiment survivors were killed and examined. This procedure was adhered to in all subsequent experiments.

The results showed that in Group I, 18 turkeys died from blackhead. Of the five survivors, two showed evidence of blackhead and three were negative after four weeks ; this represents an infection rate of 87 per cent. In Group II eleven turkeys died from blackhead, while all 12 survivors were negative, *i.e.*, an infection rate of 49 per cent. Only one turkey died from blackhead in Group III, the remaining 22 being negative, *i.e.*, 4 per cent. infection.

Artificial Infection by Means of Cultures

The inocula were cultures of *Histomonas meleagridis* isolated from the livers and caeca of infected turkeys and subcultured on to Boeck and Drbohlav's medium twice weekly. They were not pure, all of them being contaminated with bacteria and some containing *Trichomonas gallinarum*.

Two one-month-old turkey poults were used for each culture and all birds were kept in cages of a laying-battery type free from draughts and infected material. Immediately before inoculation all poults were given an enema of 5 to 10 c.c. of sterile Ringer's solution. They were then injected rectally with 2·5 c.c. of a 3-days-old subculture. Eighteen cultures in all were inoculated into a total of 36 poults. Two poults were kept as controls and they received 2·5 c.c. of sterile Boeck and Drbohlav's medium only. Only two cultures, originally isolated from caeca, produced blackhead in the inoculated poults. The remaining 32 poults were all negative.

The poor results obtained by this method compare unfavourably with the records of other workers and may be due to the fact that seven of the cultures had been subcultured for three to four months prior to inoculation. In this connection it is interesting to note that the positive results were obtained by means of freshly isolated cultures, although on the other hand several other cultures of comparable age produced negative results.

In view of the large amount of work involved in maintaining cultures for the purpose of infecting turkeys, it was decided to try the alternative method of using infected organs from cases of blackhead as inocula in place of cultures, and accordingly the following series of experiments was carried out.

Artificial Infection by Means of Diseased Tissue

The object of this experiment was to determine the optimum conditions for artificial infection by injection of infected organs, with particular reference to (*a*) source of material, and (*b*) method of injection.

Material was obtained from turkeys known to be infected with blackhead; some of these were dead birds and others showed clinical symptoms and were killed immediately before autopsy. The livers and caeca were finely chopped with scissors and emulsified separately in Ringer's solution. These emulsions were injected into poults, six birds being used for each sample of liver and caeca from an infected bird. They were starved on the day of injection and two were given enemas a few hours previously and again immediately before injection. Each group was kept in a separate cage. Eight uninjected birds of comparable age and weight which were kept under conditions similar to the above experimental birds, did not become infected with blackhead.

TABLE II

ARTIFICIAL INFECTION BY MEANS OF DISEASED TISSUE

Method	No. of poults used	No. of deaths from blackhead	Per cent. infection
Liver given orally	16	0	0
„ „ rectally	16	7	43·75
„ „ „ with enema	16	8	50·00
Caeca „ orally	15	1	6·60
„ „ rectally	16	13	81·25
„ „ „ with enema	16	14	87·50

The results which are presented in Table II indicate that caecal lesions injected rectally following an enema appeared to be the most efficient method of artificial infection, but a further experiment was carried out in order to confirm the results obtained, using caecal lesions as inoculum. In all cases material was obtained by removing the caeca of turkeys known to be infected with blackhead. Twenty-four four-week-old poults were used, two birds being injected from each sample of caecum. All birds were starved on the day of injection and received an enema of 5 c.c. of Ringer's solution immediately before injection. The caeca were emulsified in Ringer's solution and 2·5 c.c. injected rectally into the poults. The general procedure was the same as in the previous experiment. Positive results were obtained in 20 out of 24 cases, which represents a percentage of 83·3.

There was evidence that two birds should be used for each experiment as in two of the above groups a positive result was obtained with one bird and a negative result with the other ; on only one occasion were both birds negative. It was noted that in negative cases the inoculum was obtained from dead birds. Material for injection should, therefore, be obtained from a freshly killed turkey.

The next experiment was an endeavour to improve the efficiency of the technique for infection by rectal inoculation. Seventy-eight four-week-old poults, which had previously received one enema, were injected by the same route as in previous experiments. They were divided into three groups on the basis of the volume of inoculum used and the time interval between injections. The first group received 2·5 c.c. each on two occasions with an interval of five minutes between injections ; the second group received 2·5 c.c. each on two occasions with an interval of two hours between injections ; and the third group received 5 c.c. each as a single injection. The general procedure for previous experiments was adhered to.

In Group 1, 23 poults died from blackhead, the three survivors being negative at autopsy after four weeks. This represents an efficiency of 88·5 per cent.

In Group 2, 25 of the 26 poults died from blackhead. The remaining bird died from another cause two days after injection and was excluded from the experiment as the period was not long enough

for blackhead infection to have developed. With the exception of this bird the results obtained by this technique were entirely successful. In Group 3, 24 poults died from blackhead, the two survivors being negative at the end of four weeks. This represents an efficiency of 92·3 per cent.

Using 2·5 c.c. rectally on two occasions with two hours' interval, a further group of 26 birds was injected, the general procedure for previous experiments being adhered to. All poults died within 14 days of injection and showed typical liver and caecal lesions. These results confirmed the efficacy of the method.

STUDY OF DEVELOPMENT OF ARTIFICIALLY PRODUCED BLACKHEAD LESIONS

In view of the successful results obtained by the rectal injection of emulsified caecal lesions, it was decided to use this technique to investigate the progress of the experimental disease. Accordingly, twelve four-week-old poults were selected, and eleven of these were injected according to the technique described above. The uninoculated poult was killed on the first day of the experiment to provide a normal liver and caecum. On the third day, one injected poult was killed and one on each successive day thereafter. This system worked admirably up to the eighth day, but on the ninth three birds died from blackhead, thus leaving only one survivor to be killed on the tenth day. All inoculated birds showed evidence of blackhead and the appearance of the liver and caecum in each case, including those of the control bird, is shown in the accompanying photographs (Fig. 1). The liver and caeca in the control bird were normal. One of the caeca of the bird killed on the third day had a small haemorrhagic patch at the extremity of the lower segment but the liver was normal. The bird killed on the fourth day exhibited no abnormality in the liver but the caeca showed extensive haemorrhage and swelling with early necrosis and some thickening of the caecal wall. On the fifth day the liver appeared normal but the caeca showed considerable enlargement with thickening of the wall which had small caseous patches adhering to it. On the sixth day the liver had numerous pin-point lesions while the caeca were enlarged and almost completely filled with caseous material. On the seventh day the liver lesions were slightly larger, with one or two assuming a more necrotic appearance. Both caeca had a haemorrhagic area with enlargement and a hard caseous core. The bird killed on the eighth day showed a number of well-defined lesions in the liver, the caeca presenting a similar appearance to that seen on the seventh day. All birds that died on the ninth day were of similar appearance to the poult that was killed on that day. The liver showed numerous advanced whitish lesions with some haemorrhage in the liver, while the caeca were grossly enlarged and filled with a spongy fibrous network. The remaining bird, which was killed on the tenth day, had large indurated

necrotic lesions and numerous smaller lesions in the liver. The caeca showed a similar appearance to that observed on the ninth day.

The picture presented here is not complete and more precise information is required on the progress of the liver lesions between the fifth and sixth days. Furthermore, histological examinations of the liver and caeca will be necessary. It is not, however, within the scope of this article to enlarge on this aspect of the problem as this work is in progress and it is hoped to publish the findings at some future date.

DISCUSSION

The foregoing experiments illustrate clearly that it is not a difficult matter to produce blackhead artificially and much of the confusion reported in the literature might have been avoided if the methods of infection used had been more reliable and less open to doubtful interpretation. It has not been possible to obtain more than 87 per cent. infection by running turkeys on infected ground along with poultry, and as this allows for a considerable margin of error the alternative method of artificial infection is recommended. This has given consistently good results throughout the series of experiments and with certain improvements in technique an incidence of 100 per cent. was achieved. Fifty-one infections have been obtained in the poults inoculated by this method.

Although no opinion is ventured as to the exact nature of the causative agent contained in the inoculum, the following technique is recommended as giving consistent results. The experimental birds should be young turkey poults four to six weeks old, housed in cages of the laying-battery type and kept off the ground away from draughts and possible infection from other sources. Infected material for injection should be obtained from a turkey suffering from blackhead. The bird should be killed and the caeca removed and emulsified in Ringer's solution. The experimental birds should be given an enema of 5 c.c. of Ringer's solution prior to injection and 2·5 c.c. of the caecal emulsion is then injected rectally. The injection should be repeated after an interval of two hours. A 5 c.c. syringe is quite satisfactory when used in conjunction with a piece of rubber tubing of suitable bore about six to eight inches in length. At least four inches of tubing should be passed into the rectum at the time of injection, and in order to ensure that all the inoculum passes into the intestine, it is advisable to inject 5 c.c. of air immediately afterwards.

Caecal lesions can be demonstrated from the third to the fifth day, and death takes place from the ninth to 14th day after injection. The liver lesions are not so well defined as in spontaneous blackhead occurring in older birds, but appear as small, round gray patches in the liver from the sixth day after injection. The caecal lesions are, however, more advanced and necrotic than in natural blackhead and the caeca themselves are grossly enlarged.

The method is recommended as suitable for infecting turkeys to be used for experimental work in connection with the testing of drugs for prophylactic or therapeutic purposes.

SUMMARY

A method is described for the artificial infection of turkey poults with blackhead. Material for infection is obtained from live infected turkeys by removing the caeca, which are emulsified and injected rectally into four-week-old poults. The experimental birds die within nine to 14 days after infection.

Photographs of the progress of the disease from the day of infection are included.

ACKNOWLEDGMENT

We would like to take this opportunity of recording our indebtedness to various colleagues who have offered helpful advice and who have made it possible to carry this work to a successful conclusion. In particular we would thank Mr. J. Bennett who reared and supplied all turkeys used, Mr. G. Whiting for his excellent photographs, and Miss P. Caunt for her invaluable technical assistance. We would also express our thanks to Miss Bishop for advice on the artificial culturing of *Histomonas meleagridis* and to Sir Jack Drummond for his permission to publish the results of this work.

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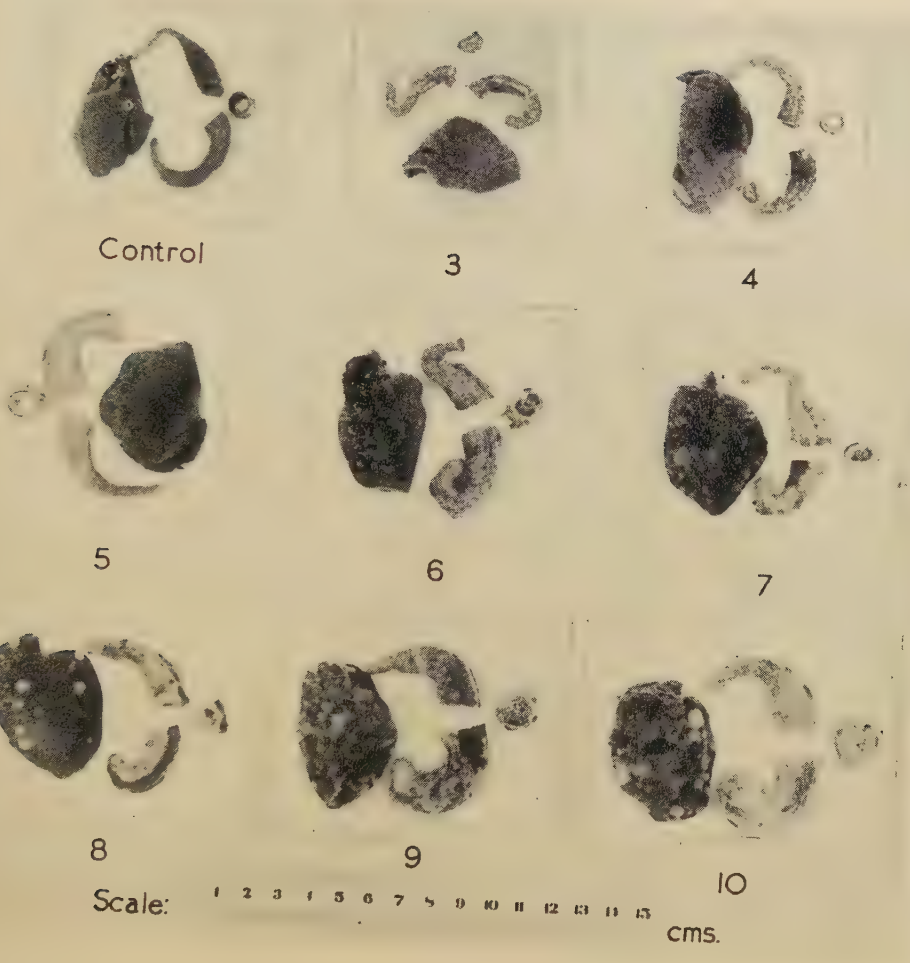
FIG. 1.



CAECOSTOMY IN THE HORSE

By

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INTRODUCTION

Although the digestive system is frequently involved in disorders of the horse, there is little information available about the mechanics of the gastro-intestinal tract in this species. Indeed, other than the early observations of Colin (1886) and the studies on stomach movement of Schalk and Amadon (1921), the problem seems to have attracted few students. The size and inaccessibility of the abdominal organs and the difficulties attendant upon surgical intervention in this region have undoubtedly deterred such investigations. With a view to overcoming some of these difficulties, the possibility of preparing permanent fistulae into the large intestine was explored, and a satisfactory technique evolved.

TECHNIQUE

The operation was performed in two stages. The first involved the formation of a peritoneal ring and the exteriorisation of a portion of the caecum; the second, performed at a later date, entailed the opening into the caecum and the completion of the fistula.

Pre-operative Treatment.—Food was withheld for the 36 hours prior to operating and no water given in the last twelve hours of this period. An extensive area on the right flank was shaved, thoroughly washed and cleansed with spirit 24 hours before the operation.

Restraint.—The animal was cast on its near side with side-lines and the anaesthetic solution injected into the right jugular vein by gravity. When anaesthesia was complete, both fore and the near hind legs were secured together, and the off hind drawn backwards with a single rope. This position was maintained until the final stage of the operation that of suturing bowel to skin, when the off hind leg was drawn forwards and secured to the others.

Anaesthesia.—Chloral hydrate, made up in 10 per cent. solution with 1 per cent. sodium citrate, was used to induce and maintain anaesthesia, the initial dosage being calculated on the basis of 7 g. chloral per cwt. body weight. Further injections of 100 c.c. were given when deemed necessary to maintain the required depth of anaesthesia.

Operation

The site was swabbed with spirit, then with iodine solution, and towels applied. An incision was made through skin and subcutaneous tissue, commencing 2 in. below the mid-point of the line joining the coxal tuberosity to the head of the last rib and extending downwards and slightly forward for a distance of 6 in. Towels

were then applied to the lips of the wound and the incision deepened through the aponeurosis of the external oblique abdominal muscle, exposing the thick internal oblique abdominal muscle. This muscle was incised across its fibres in line with the primary incision. A considerable oozing of blood resulted, but only one major vessel, a branch of the circumflex iliac artery, was encountered. The lips of the wound were then strongly retracted to expose the fascia of the transverse abdominal muscle. The fibres composing this fascial sheet were split with scissors to within ½ in. of the commissures and the exposed peritoneum secured by Allis forceps at the top and bottom of the incision and two points midway; following traction on the central pair of forceps, the peritoneum was opened along the line of the wound.

The next step was to form a peritoneal ring by suturing the peritoneum to the outer edge of the internal oblique muscle. Interrupted sutures of 00 silk on straight needles were placed ¼ in. apart. A good "bite" of peritoneum and muscle was taken and special attention was paid to the upper and lower commissures to ensure their being fully covered.

The caecum was then located and completely withdrawn from the abdomen to ensure identification. An area on the lateral surface at the junction of the base and the body, was selected for exteriorisation, the remainder of the organ being returned to the abdomen. The retained portion was firmly secured to the peritoneal ring by interrupted sutures of 00 silk placed ½ in. apart, care being taken to obtain good apposition of peritoneal and serosal surfaces. The sutures included the caecal mucosa within their grasp and were so placed that a considerable portion of the caecal wall protruded from the lips of the wound. As before, the commissures received special attention. Finally the caecum was sutured to the lips of the skin wound by through and through mattress sutures of heavy linen. The serosal surface of the caecum was thus brought into contact with edges of the skin wound.

The area was thoroughly smeared with sterile vaseline and a similarly treated Gamgee pad stitched over the exposed bowels. This dressing was changed daily until the second stage of the operation.

Second Stage.—A considerable reaction took place on the exposed serous surface, with marked exudation and great thickening of the bowel wall. Granulation tissue soon appeared, and after five and a half days the serosal surface was firmly adherent at most points to the edges of the skin wound. The second stage was then undertaken.

General anaesthesia was again induced with intravenous chloral hydrate and the animal secured in a recumbent position. The whole area was then thoroughly washed with soap and water, swabbed with normal saline solution and cloths applied. After the removal of excessive granulations, the caecal wall was picked up with forceps and slit

FIG. 1.—(*a*) Caecum exposed at operation. (*b*) Caecum exteriorised. (*c*) Fistulae three months after completion.

(*a*)

(*b*)

FIG. 2.—(*a*) Inflatable rubber stopper. (*b*) Stopper *in situ*.

with scissors along its midline to within ¼ in. of upper and lower commissures. A moistened towel was then quickly inserted to pack off the caecal contents. Close apposition of the lips of the caecal wound and the skin edge was then obtained, using interrupted sutures of No. 7 nylon. A firm hold of skin and caecal wall was taken, the sutures being placed ½ in. apart. Hot packs wrung out with saline were applied constantly throughout. The special "stopper" was next inserted and securely seated. It was inflated to moderate tension and the edges of the outer plate attached to a surcingle passing round the back and loins.

At first there was a good deal of inflammatory reaction around the fistula with considerable serous exudation and formation of granulation tissue. Owing to leakage of caecal contents it was impossible to avoid contamination of the wound and some surface infection resulted. By the seventh day from completion of the second stage, the mucosal surface had firmly united with the skin edge at all points. From then on the reaction quickly subsided, the mucous membrane showing a moist healthy surface. Three weeks from completion of the second stage, the epithelium at the fistula edges was commencing to show a squamous character.

A slight post operative rise in temperature and pulse rate was noted 48 hours after the first stage, but this had subsided by the 72nd hour. At no other time was the temperature or pulse abnormal, nor did the animal refuse food. Various steps of the operation are illustrated in Figs. 1 and 2.

THE INFLATABLE STOPPER

The form of apparatus finally employed for closure of the fistula consisted essentially of two lozenge shaped soft rubber plates joined by an oval inflatable tube. A photograph of the apparatus is shown in Fig. 2. The outer plate measures 8 in. in its vertical diameter and 6 in. in its greatest horizontal diameter and is moulded in ¼ in. rubber. The inner plate is 1 in. less in overall measurement and of the same material. The distance between the two plates is 3 in. The inflatable tube is attached to the plates 1½ in. from their outer margins. For experiments which entail frequent manipulations of the fistula as, for example, the hourly sampling of caecal contents, it is convenient to have the stopper fitted with a central tube connecting the rubber plates and closed with a rubber stopper.

DISCUSSION

The problem presented two main aspects, the exteriorisation and opening of the large bowel without peritoneal soilage and, having made a fistula, the designing of some means of preventing leakage of ingesta. The surgical technique entailed fewer difficulties than was at first expected in view of the current teaching regarding abdominal surgery in the horse. Chloroform anaesthesia was used

for the first experiments, but prolonged anaesthesia necessary for the operation caused pneumonia; intravenous chloral hydrate anaesthesia avoided this complication. A starvation period of 36 hours was found to be the minimum time necessary to obtain a relatively empty caecum.

It was considered that the main factor in preventing peritoneal contamination was the careful preparation of the peritoneal ring so as to secure adhesion between serosal and peritoneal surfaces. The horses tolerated this interference surprisingly well and, in recent experiments, the provision of a fistula has not caused the animal to miss a single feed.

The opening into the caecum should be delayed until the reaction has subsided and complete union achieved between the opposed surfaces: this was usually found to have occurred by the fifth or sixth day. Much more difficulty was encountered in designing a suitable apparatus for closing the fistula. In the early experiments a large tracheal cannula was used, but this only allowed a relatively small opening and permitted extensive leakage. Further experiments with a variety of metal cannulae of this basic design proved unsatisfactory. The idea of having a solid type cannula was abandoned and an inflatable rubber stopper devised. The design finally selected closed the fistula effectively, prevented leakage of the air and ingesta and did not damage the mucosa. Horses fitted with this type of apparatus showed no loss of condition over several months.

Fistulae of the type described should prove valuable in physiological and pharmacological studies. The rubber stoppers could be modified for various species and should prove more satisfactory than the conventional types.

SUMMARY

A method has been described for the preparation of permanent fistulae into the large intestine of the horse.

An inflatable rubber stopper was devised which prevented leakage from the fistula.

ACKNOWLEDGMENTS

We wish to take this opportunity of thanking Mr. and Mrs. Allison, of Norton Mains, for the facilities to conduct these experiments, Mr. Robin Allison for the care taken to secure the photographic records and the Agricultural Research Council for a grant to one of us (F.A.) which allowed this work to be performed.

The inflatable stoppers were made by the North British Rubber Company, Edinburgh, and we are grateful for the interest shown by Mr. McGowan of this company.

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SPECIFIC AND NON-SPECIFIC DESENSITISATION OF THE SKIN IN TRICHOMONAS SENSITIVE BOVINES

By

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INTRODUCTION

In a series of studies on antibody response in cattle to infection and vaccination with *Trichomonas foetus*, we were able to show that fasting newly-born calves developed a serum titre a few hours after they had ingested colostrum containing antibody against trichomonas (Kerr and Robertson, 1946). This passively acquired antibody is followed in the course of some varying number of days, usually four to ten or more, by the appearance of the positive skin reaction to the intradermal injection of extract of trichomonas. The skin reaction has been observed in one case as early as 15 hours after the ingestion of three pints of high titre colostrum.

In our view the skin reaction is due to a local response to the junction of antibody and antigen in such a way as to cause a temporary collection of fluid and the production of a bleb. We consider that the antibody is lodged somewhere in the dermal and epidermal layers of the skin. There is evidence in our experimental work that the skin (total hide) of a sensitive animal will yield on extraction with saline the specific agglutinin for *Tr. foetus* and also that the same treatment of the skin of a normal animal does not. In these tests the blood content of the undiluted extract on titration against diluted blood by the usual colorimetric method was less than 1 : 200 (0·5 per cent.). The highest concentration of extract used was 1 : 10 so that the blood content in the first dilution was at most 1 : 2,000. As the end point of the agglutination reaction in the extract was positive in 1 : 100 the blood dilution would be more than 1 : 20,000. The end point of the agglutinin in the serum of the animal (D14) was positive in 1 : 1,500. This seems to exclude the capillary blood that might be left in the hide after scraping and washing as the source of the agglutinin. It does not, of course, exclude the lymph as a source

* Part of the work was carried out while one of us (M.R.) was in receipt of a grant from the Agricultural Research Council,

of the antibody nor does it give any clue as to the actual site of the location of this substance in the dermis or epidermis.

The quantitative titration of antibody against *Welchii* toxin from various tissues has been carried out lately by Keppie and Macfarlane (1948), and they have kindly permitted us to quote some of their figures. They found that antibody could be demonstrated in the actively immunised rabbit in the heart muscle, liver, lung, skin and uterus but not in the skeletal muscle or the brain. The antibody fixed in each of these various tissues amounted to about 5 per cent. of the serum titre. The conditions of this work make it also unlikely that the capillary blood is the source of the antibody.

In a normal animal never exposed to Trichomonas the antigen produces no disturbance by itself when injected intradermally. In a sensitised animal which reacts it seems justified to conclude that the junction of antibody and antigen produces a new substance which causes the collection of fluid and the disturbance registered as an " intradermal reaction " or bleb. When an animal has antibody circulating in the blood stream and it does not respond to the intradermal injection of antigen, the animal is said *not* to be skin sensitive or to be desensitised. The greatest care must be taken, however, as to what conclusions are drawn from this absence of reaction. It may mean a temporary denuding of the skin of antibody, but that is not an inevitable deduction. It might mean either that there is a junction of antibody and antigen when the animal is not in a state to produce the manifestation, *i.e.*, the collection and retention of the fluid to cause the bleb, or that the reaction is inhibited by some other condition taking place in the body. It might mean also that the junction of antigen and antibody is no longer a substance with the disturbing character.

At this stage of our knowledge, the analysis of these problems can be approached from several angles. The present study deals with the recording and to some extent with the elucidation of the conditions producing and accompanying desensitisation without prejudice as to whether the physiological processes involved are always the same. It is impossible to approach any of the problems of sensitisation or desensitisation without reference to the immune processes going on co-incidentally in the body.

In the last 20 years work on the animal response to the presence of antigens has brought out what an important part the lymphocytes and the lymphatic system play in the production of antibody (Erich and Harris, 1942 ; Harris, Grimm, Mertens and Erich, 1945). The work of Chase, White and Dougherty (1946) and Dougherty and White (1944, 1947), White and Dougherty (1946) has directed attention to the white blood cell reaction to the presence of hormones of the adrenal cortex-pituitary complex and the similar reaction to Sphingomyelin has been recorded by Tompkins (1946). These two trends in research appear to be heading in the same direction, at least in certain aspects of the work, and both would seem to have a

bearing upon the problems of immunity, sensitisation and desensitisation.

The work recorded deals primarily with the macroscopic reaction of the animal but the differential and total white cell counts have also been followed in selected examples.

METHODS AND MATERIAL

The animals used were sensitised in various ways. They were vaccinated in some cases intramuscularly and in others by instillation of antigen into the non-gravid uterus of calved cows. Animals sensitised by infection were also included. Some of the experiments were done with calves passively sensitised by feeding on colostrum.

The antigens used, apart from living suspensions, were freeze-dried whole bodies and acetone-dried whole bodies. In some cases trichloracetic acid precipitates of organisms laked in distilled water were used, but this antigen has disadvantages in the preparation which limits its value and it has not been used to any great extent. The blood tests were carried out in the manner fully described in earlier papers. (Robertson, 1941 ; Kerr and Robertson, 1941 ; Pierce, 1947*a*.)

In the earlier work the skin testing fluid was made from trichloracetic acid precipitates of laked organisms. In all the later work and in all the repetition of important findings, a non-antigenic ethylene-glycol extract was used. This was prepared by Dr. Morgan and Dr. Feinberg and will be reported upon in due course by these workers. This substance has been of very great value in the investigation as its use caused no desensitisation even when the tests were repeated daily. The absolute specificity of the extract has not been established as yet in field trials, but when used in animals of known history and with proper control tests before the inauguration of immunisation, it has proved to be very valuable and reliable. Skin reactions showing a rise up to 2 mm. inclusive were considered to be negative, 2·5 mm. was held to be a borderline negative and 3 mm. to 3·5 mm. a low positive : everything above that was read as a positive.

Blood Counts.—The total white cell counts, which were all recorded by the same observer (J. L. McG.), were made in citrated blood in the Thoma Zeiss Haemocytometer or in the Neubauer counting chamber. The mean of two counts was taken as the final figure. The differential counts were prepared from blood films made directly from the animal. They were stained by Giemsa's method and 200 cells were counted as a routine. The counts for any series were usually made by one observer (M. R. or J. L. McG.). Important counts and any discrepancies were checked by further counts by both observers.

It is important to note that the graphs are drawn from the total differential counts. These figures are essential in order to appreciate the true significance of the changes produced. The percentages alone do not reveal the full extent of the important variations in absolute numbers.

Sphingomyelin and Adrenal-cortical-steroid Solution.—The sphingomyelin which was prepared by one of us (J. L. McG.) according to the method of Levene (1916) is obviously not a pure substance but in the circumstances it was not possible to obtain the adrenal cortical hormone in a sufficient supply for free experimentation. The action of sphingomyelin on the white blood

cell count is very similar to that of the adrenal-cortical steroid solution (Tompkins, 1946 ; Dougherty and White, 1947 ; Chase *et al.*, 1946).

A supply of the lipo-adrenal cortex steroid preparation made by the Upjohn Company was kindly obtained by the Medical Research Council and placed at our disposal. It was used in a small number of carefully planned experiments.

OBSERVATIONS AND EXPERIMENTAL DATA

The conditions bringing about desensitisation dealt with here, fall into three groups. (1) Loss of sensitivity by passage of time ; (2) desensitisation brought about by the absorption of specific antigen in one way or another ; (3) non-specific desensitisation by parturition and by the injection of (*a*) sphingomyelin, and (*b*) steroid solution of adrenal cortical extract.

Loss of Sensitivity by the Passage of Time

This type of loss of sensitivity can be seen very clearly in the disappearance of the skin reaction in calves passively sensitised by the ingestion of antibody in the colostrum during the first 24 hours of life.

Out of many examples B.17 c, (1943) was chosen because here the calf was the offspring of a normal mother and it had received a strictly limited amount of colostral whey containing antibody. The whey was derived from the colostrum of B.15, a cow parenterally injected with *Tr. foetus* antigen during pregnancy. The amount of whey given was 1½ pints with 1½ pints of boiled milk. The whey of the milk was tested for antibodies and was negative. The agglutination index of the whey from B15 was 16½/9½ which is a good average, but not the highest possible antibody content.

The blood of the calf before feeding showed no agglutinins at all. Twenty-four hours after feeding the index of the blood was 9½/9. The skin reaction showed a rise of 4·5 mm. (positive) 39 days after the sensitising feed. No earlier test was made. Four months later the skin reaction was still equal to a rise of 4·5 mm. and was registered as positive. From the end of the fourth month till the end of the seventh month the skin reaction lay within the doubtful range but by the end of the eighth month it was definitely negative and remained consistently so from then onwards.

In this animal the blood titre of passive antibody was never very high though clearly present. It is, of course, not possible to ascertain the actual moment of disappearance of the circulating specific antibody owing to the developing normal non-specific titre in the calf. In the adult this period is also obscured by the non-specific normal titre remaining constant at a time when the antibody has sunk below the detectable level (Kerr and Robertson, 1941).

The time at which the skin test becomes negative in calves passively sensitised by means of the colostrum varies greatly. It bears, however, some relation to the amount of antibody in the ingested

colostrum. The longest time of which we have accurate data is about 13 months, but this is probably not the maximum duration of passive sensitisation.

The skin sensitiveness after an infection with living organisms gradually fades but no accurate details have been obtained. The blood titre declines but its duration is very variable. In some field cases it has lasted for at least 13 months (case 72).

Desensitisation by time alone in cases of sensitisation of the skin by intramuscular or subcutaneous injection of antigen is again very variable. It depends on the capacity of the animal to respond to the vaccination and to fix the antibody. In these experiments where a good agglutination titre of considerable duration has been recorded the skin reaction is both more definite and, as would be expected, is of longer duration. It should be noted, however, that some animals which develop antibody in the circulating serum showed a poor and varying degree of fixation in the skin. This type of fluctuating reaction is a character of certain animals as other virgin heifers treated in exactly the same way produced extremely tenacious skin reactions. The exact duration of a good skin response after vaccination has not been observed as the animals could not be kept for that alone and were used in other experiments, but the skin can remain sensitive in certain animals after infection or vaccination for at least a year.

Desensitisation by Absorption of Specific Antigen

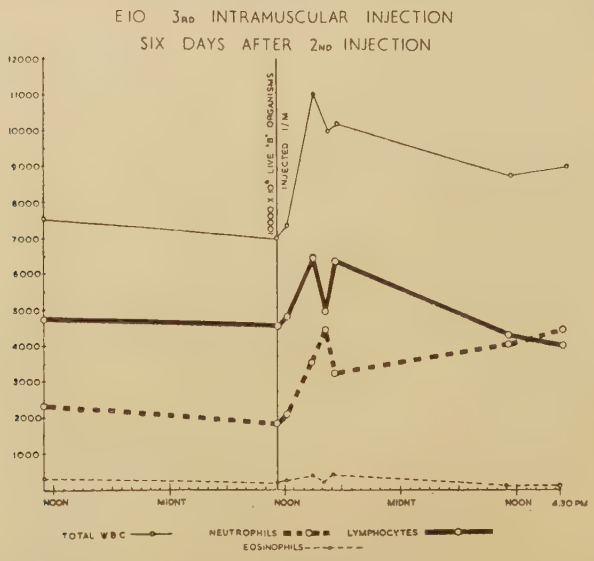
(*a*) *Desensitisation of the Skin by Parenteral Injection of Antigen.*—This was a very frequent effect. A clear case of this type of desensitisation where the reaction of the white blood corpuscles was followed occurred in the bullock E10.

The animal received three closely spaced intramuscular injections of living organisms (strain Belfast) on 7.4.48, 12.4.48 and 20.4.48. This produced a blood index of $17\frac{1}{2}/9$. The skin was sensitised during the immunisation.

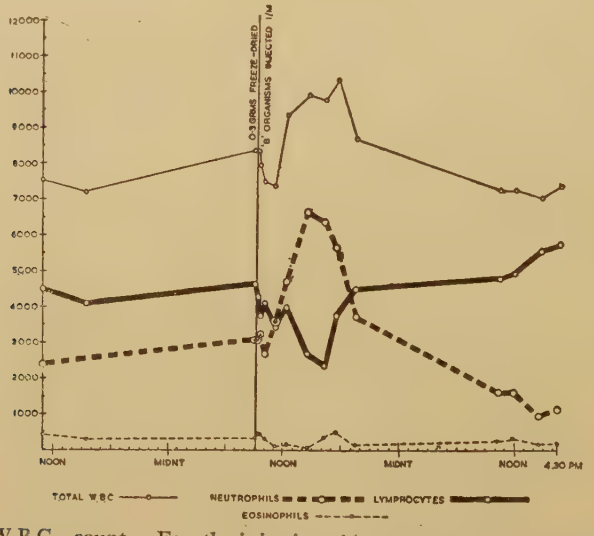
Twenty days after the third dose the skin reaction equalled a rise of 7 mm. On the following day the animal received an intramuscular injection of 0·3 mm. of crude freeze dried organisms (strain Belfast) at 9.05 a m. At 4.15 p.m. the skin test showed a rise of 3 mm. (low positive). Subsequent tests were as follows : one day, 1 mm. (negative) ; two days, 2 mm. (negative) ; eight days, 3 mm. (low positive) ; ten days, 5 mm.

The white cell response was followed in this animal and the reactions to the third and fourth injections are reproduced in graphs 1 and 2. The total white cell count ran pretty steadily between 6,500 and 8,500. Immunisation, especially the first two doses, produced white blood corpuscle reactions characterised by a rise in the total count reaching its maximum at about the fourth to fifth hour after the injection. After the first injection the rise involved only the neutrophils, but after the second injection both lymphocytes and

neutrophils were affected. After the third injection both sets of cells showed a rise in number (Graph 1). The lymphocytes show a rise



GRAPH 1.—Differential W.B.C. count. Third injection of antigen intramuscularly. (E10.)



GRAPH 2.—W.B.C. count. Fourth injection i/m 21 days after third injection. Note fall in lymphocytes and rise in neutrophils. Skin is temporarily desensitised. (E10.)

which fluctuates a little and lasts for about six hours, returning to the pre-injection figure in 24 hours. The neutrophils show a rise which declined by the sixth hour but the numbers remained a little

high on the second day. The skin test on the morning of the injection was equal to a rise of 5 mm. (positive); on the morning after it was 3·5 (low positive). The reaction had been reduced but the animal was not completely desensitised.

The cell response after the injection of dried antigen which was given 21 days later and which desensitised the skin is shown in graph 2. Here the immediate reaction is a slight drop in the total count. The lymphocytes declined in numbers with slight fluctuations (which cannot be considered significant) until the seventh hour, and then rose during the eighth to tenth hours to the pre-injection level. The decline in the lymphocytes was quite marked. The neutrophils which were only faintly involved in the early slight drop of the total count began to rise between the second and third hour and remained markedly high and above the lymphocyte count until the tenth hour. The total count, which rose from the second hour and was still above the pre-injection level at ten hours, reverted to the normal level at 24 hours. The lymphocytes had by this time returned to the pre-injection figure but rose a little during the second day, returning to normal in 48 hours. The neutrophils were somewhat low during the 24-hour to 36-hour period but were back at the normal level at 48 hours. This crossing over of neutrophils and lymphocytes, and particularly the drop in the level of the lymphocytes, appears to be a significant feature of the cell reactions accompanying desensitisation in these circumstances.

These examples could be multiplied with some variation in the rapidity of the desensitisation which is often noted in 24 hours and in the interval before resensitisation occurs.

(*b*) *Desensitisation Through Multiplication of Trichomonads in Uterus.*—Desensitisation may arise during an acute infection when there develops an effusion of fluid into the uterus in which trichomonads multiply in very large numbers ("Pyometra"). The following are two good examples which occurred in animals in which acute infections were set up by experimental infection with strain Belfast at insemination.

D6—virgin heifer vaccinated with living organisms.

11.9.44: Blood index 19½/9; skin reaction = rise of 8 mm.; last vaccinating dose given 14 days previously.

30.9.44: Exposed at insemination to *Tr. foetus* and became infected. The skin reaction was positive throughout this period.

12.1.45: The skin showed a rise of 9·5 mm.

17.1.45: There was a profuse watery discharge crowded with trichomonads. The uterus was very much enlarged and in the state often diagnosed as "trichomonas pyometra." The skin showed a rise of 2 mm. (negative).

18.1.45: Trichomonads were still very numerous in the discharge. The skin reaction increased by 2·5 mm. (borderline negative).

19.1.45: The uterus was reduced in size but was still fluctuating; trichomonads were numerous in the discharge. The skin showed a rise of 4 mm. (positive).

20.1.45 : The uterus was still smaller but was not evacuated. The skin showed a rise of 3 mm. (low positive).

23.1.45 : The calf was palpated ; skin showed a rise of 4 mm.

28.1.45 : A foetus with its membranes was aborted. The discharge was scanty but trichomonads were numerous.

30.1.45 : The blood index was 17/9. The uterus was small and firm and trichomonads had disappeared. The skin showed a rise of 6 mm.

2.2.45 : The skin reaction increased to 10 mm. The blood index was 17/9.

Here is an example of the desensitisation of the skin at the time of the formation of the effusion into the uterus as shown by the swelling of the organ. The discharge at this time was full of trichomonads which must have been multiplying very rapidly. This desensitisation is, in our view, due to the absorption of antigen and to the conditions set up by this process and is similar in general root character to that described above in the section on desensitisation of the skin by parenteral injection.

The *act of abortion* did not here inaugurate the desensitisation as this had taken place eleven days before the abortion.

D7—virgin heifer vaccinated with live organisms.

In this animal the blood titre produced by the vaccination had a tendency to drop more quickly than is usual and the skin desensitised rather easily.

30.10.44 : The animal was successfully infected at insemination with *Tr. foetus* (strain Belfast). The skin test was negative. The skin reactions were as follows : 1.12.45, 3 mm. (low positive) ; 2.1.45, 5 mm. (positive) ; 12.1.45, 5 mm. (positive).

31.1.45 : The right horn of uterus was much enlarged and a diagnosis of " pyometra " was made.

2.2.45 : The blood index was $15\frac{1}{2}/9$ and the skin showed a rise of 1 mm. (negative).

8.2.45 : There was a profuse discharge of very thick pus full of trichomonads. Four hours later a foetus 4 inches long was aborted along with its membranes, the fluid and the foetus being both heavily infected with *Tr. foetus*. The skin showed a rise of 2·5 (borderline negative).

9.2.45 : The blood index was 14/9 and the skin increased to 2·5 mm. (borderline negative). The trichomonads had disappeared.

2.3.45 : The skin reaction rose to 3·5 mm. (low positive).

The time of the abortion was $14\frac{1}{2}$ weeks from the date of insemination and exposure to infection, but the size of the foetus corresponded to about eight weeks' gestation. In this example, it was clear that the thick pus in the uterus, full of trichomonads, was external to the membranes so that there were large numbers of trichomonads between the placenta and the uterine wall. This observation is important as the exact location of the trichomonads is difficult to ascertain with certainty in most cases. In this case the desensitisation was again connected with the formation of a " pyometra " and the multiplication of the organisms ; moreover, it was clear that the organisms were in direct contact with the uterine wall.

(*c*) *Instillation of Antigen into Uterus.*—A type of desensitisation

which is in part an experimental reproduction of some of the characters of acute infection occurs when antigen is instilled into the uterus of a non-gravid cow whose skin has been previously sensitised. If the conditions and dosage are appropriate the skin will be desensitised.

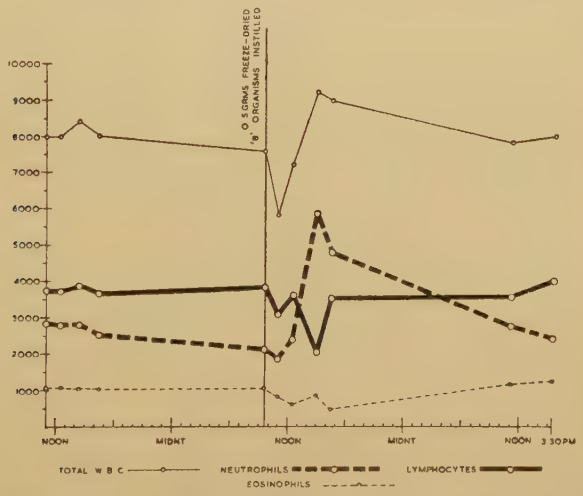
It should be noted that owing to the fact that antibody is at times localised in the uterus (Kerr and Robertson, 1947) the picture may on some occasions be a composite one, but in many cases the reaction follows in much the same way as other specific desensitisations by absorption of antigen. An example is afforded by D14. This was a normal calved cow, an animal which had been vaccinated intramuscularly and had then been sensitised by uterine instillation of crude freeze-dried trichomonas antigen. It was one of the animals which had shown a localisation of antibody in the uterus in the spring of 1947 (Kerr and Robertson).

In the present experiment four instillations into the uterus had been carried out with freeze-dried organisms on 19.11.47, 25.11.47, 16.12.47 and 31.12.47 without producing any evident local reaction in the uterus.

2.2.48: The skin reaction showed a rise of 5 mm.

4.2.48, 9 a.m.: Skin reaction showed rise of 7 mm. (35 days since last instillation); 9.30 a.m.: 0·5 gramme freeze-dried antigen in 30 ml. saline instilled into uterus; 11 a.m.: Slight local reaction. The uterus was enlarged and tense; 12.30 p.m.: The uterine reaction was passing off.

5.2.48: The skin showed a rise of 2 mm. (negative).



GRAPH 3.—Desensitisation of skin by uterine instillation of antigen. W.B.C. counts show cross-over of lymphocytes and neutrophils. (D14.)

The slight local reaction is not a necessary accompaniment of the desensitisation which may occur without any obvious symptoms. This type of desensitisation seems merely to be a special case of the effect of the absorption of antigen in a sensitised animal.

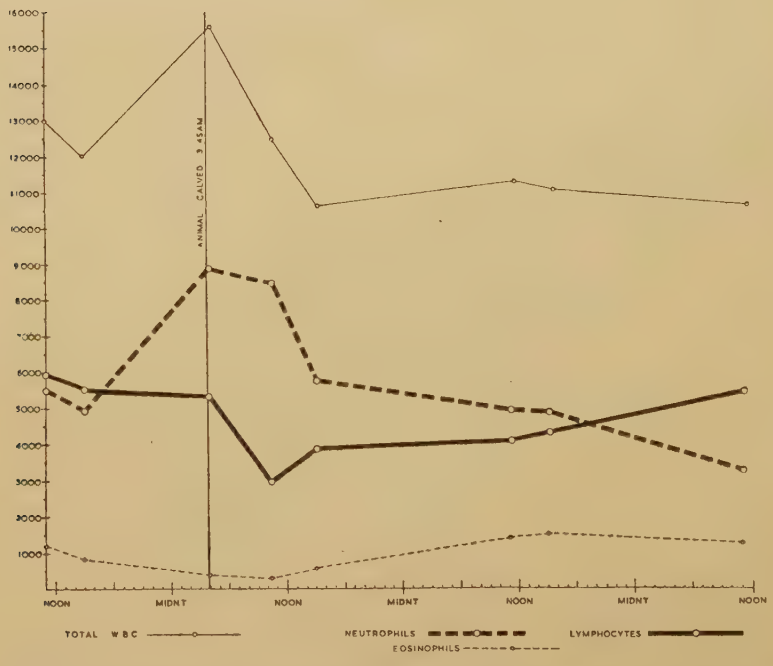
The W.B.C. counts are shown in graph 3. The lymphocytes suffered a depression in number and the polymorphs were increased. The maximum point of the reaction took place at $5\frac{1}{2}$ hours.

Non-Specific Desensitisation

(*a*) *Desensitisation of the Skin at Parturition.*—The study of trichomoniasis has afforded many examples of this type of desensitisation, but the selection for the present purpose depended upon the skin tests having been made at the appropriate intervals. This reaction had been taking place during a considerable period of the research without any attention being paid to it as our interest was centred at that time upon the transference of antibody to the calf.

D4 is an example of desensitisation at parturition which was closely observed. The animal had a long history of immunisation with trichomonas antigen and had also had an acute infection. She was vaccinated intramuscularly during this pregnancy with freeze-dried total antigen in oil. The last dose was given on 2.11.47.

The skin reaction 13 days before calving was equal to a rise of 7 mm. The blood index, on 15.1.48, five days before calving, was $17/9\frac{1}{2}$ and 12 hours after parturition was unchanged. This animal



Graph 4.—Desensitisation after calving. W.B.C. counts show fall in lymphocytes and rise in neutrophils. (D4.)

gave signs of being about to calve during at least a month before actually doing so. She calved very easily and expelled the afterbirth normally and completely. She is an example of desensitisation at parturition where there was some fluctuation and gradual loss in the skin reaction, which was very noticeable from the seventh day before calving and culminated in complete desensitisation 12 hours after calving. The skin reaction then remained below the positive level for nine days. Tested 12 days later, on the 31st day after calving with no intervening test, the reaction equalled a rise of 5 mm., which is a good positive.

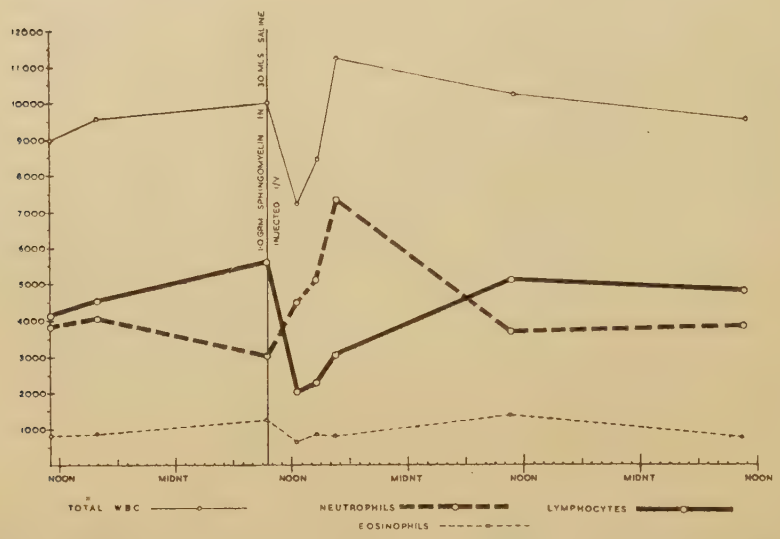
In this animal the differential white blood corpuscle counts were made over the calving period (Graph 4). For three weeks before calving there was some fluctuation in the total W.B.C. count, the lowest being 5,600 and the highest 10,000, but the relation of the lymphocytes and neutrophils retained the normal position, the lymphocytes always being more numerous than the neutrophils. Three days before calving the total count was high (13,400), and while no count was made two days before calving it was still high at 11 a.m. on the 19.1.48, 17 hours before calving. The lymphocytes and neutrophils were approximating to each other, but were still in the usual position 13 hours before calving, which took place at 3.45 a.m. A sample taken at 4 a.m. gave a high total count of 15,600, with the lymphocytes at the same figure as before but with a run up of neutrophils to a high level. Six hours later the total count was coming down but the neutrophils were still very high (8,432) while the lymphocytes had fallen to a definitely low level (2,976). The neutrophils had begun to reduce in number 12 hours after parturition, but were still high and above the lymphocytes 24 hours later, *i.e.*, the lymphocyte/neutrophil ratio, was below unity for at least 36 hours. The graph shows the gradual return to the normal state of affairs so that on the morning of the fifth day after parturition the relation of the various elements was pretty much what they had been during the earlier pre-calving period. The characteristic features of this graph are the crossing over of neutrophils and lymphocytes and the marked absolute reduction in the number of lymphocytes immediately after calving.

(*b*) *Desensitisation by Injection of Sphingomyelin and Corticosterone.*—A number of considerations led us to see if we could desensitise the skin by other physiological means. The work of Chase *et al.* (1946) induced us to consider the effect of the adrenal-cortical hormones. The suggestion that injection of antigen mobilised the adrenal-cortical-pituitary complex was the point of departure. Care was taken not to use any animals in oestrus for these experiments.

In our preliminary experiments with rabbits, using sphingomyelin, we were able to reproduce the white corpuscle reaction as described by Tompkins (1946) in these animals. Our experiments with normal cattle gave similar results. The intravenous injection of up to a gramme in saline was tolerated with only a minor dis-

turbance of respiration in the first five to ten minutes. The effect on the W.B.C. count is shown in Graph 5.

The animal (D26) was a virgin heifer, about two years old, in which the total W.B.C. count ran pretty steadily between 8,000 and 10,000. One gramme of sphingomyelin was injected intravenously



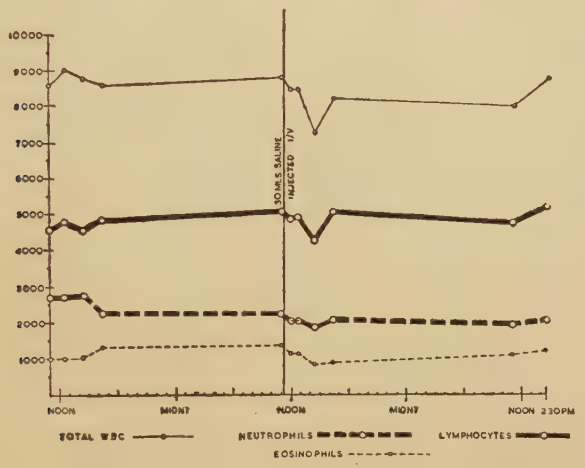
GRAPH 5.—Response of W.B.C. counts to i/v injection of sphingomyelin 1·0 g. Characteristic cross-over of lymphocytes and neutrophils. Reaction is most intense at 6 hours. (D26.)

at 9.30 a.m. The only clinical reaction produced was a slight respiratory embarrassment, which passed off in about ten minutes. In three hours there was a drop in the total W.B.C. count from 10,000 to 7,200, but the neutrophils were rising and the drop in the numbers accounted for by a striking diminution in the lymphocytes. Five hours after the injection, the total count was beginning to rise but the lymphocytes remained low. At seven hours the lymphocytes, though still low, began to rise again : the neutrophils had also risen during the period and the high number of the total count was due to the increase in the numbers of these cells. In 24 hours the conditions were returning to the pre-injection state with the lymphocytes above the neutrophils in the usual position found in bovine blood. The same animal was also injected with a saline control. There was only a slight reaction in the total counts as depicted in Graph 6.

The Graph 5 (D26) is in all essentials in agreement with the results of Tompkins (1946) working with sphingomyelin in the rabbit and with those of Dougherty and White (1947), dealing with the effect of adrenal cortical secretion. Hills, Forsham and Finch (1948), who used pituitary adrenotrophic hormone which stimulates the

production of adrenal-cortical-hormone in normal animals, described the same reaction, namely, an increase in the circulating neutrophils and a decrease in lymphocytes.

Sphingomyelin.—The sphingomyelin was always made by the same method (Levene, 1916), but it is clear that the end product

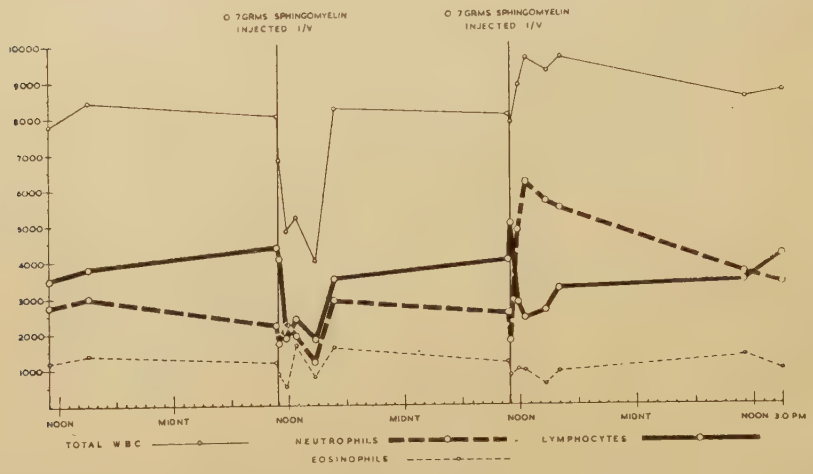


GRAPH 6.—W.B.C. counts in same animal as in Graph 5 after i/v injection of saline (30 ml.).

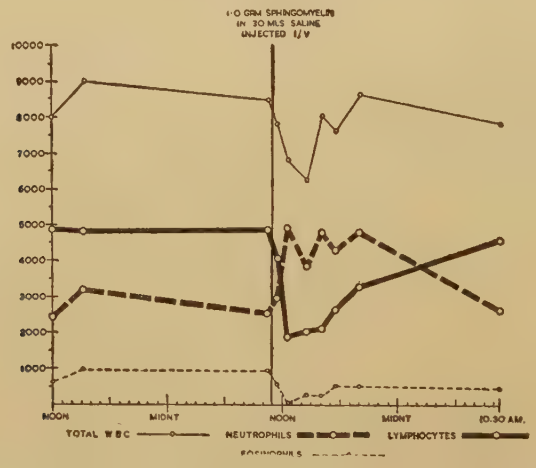
contained other elements. There was no attempt at standardisation of the substance, and it is highly probable that there was some variation in the different batches. In using the sphingomyelin made in the Stormont Laboratory, two types of white blood corpuscle response were observed: (1) the characteristic cross-over of lymphocytes and neutrophils illustrated in Graph 5 (D26); and (2) a response in which the total count suffered a reduction which lasted until after the maximum point of the reaction at six to nine hours. This type of response showed the characteristic drop in the number of lymphocytes, but there was no effective cross-over as the neutrophils did not increase in number.

Graph 7 (D10) shows both these reactions occurring in the same animal on two consecutive days with the same batch of sphingomyelin. The dose consisted of 0·7 g. given intravenously on each day. The first response was a depression of the lymphocytes and also of the neutrophils, producing a very marked drop in the total count. The cell reaction passed off quickly and the count had returned to normal by the sixth hour. On the second day after the injection of the second dose of sphingomyelin the lymphocytes again showed a considerable diminution but the neutrophil numbers rose steeply giving the expected cross-over of these cells. The total counts rose to a moderate extent. The high neutrophil count seemed to have persisted through the night as at 11 a.m. the following morning they

were still slightly high. The skin reaction was equal to a rise of 5·5 mm., which is a good positive before the first injection of sphingomyelin, but six hours afterwards the reaction showed only a rise of 2·5 mm., a borderline negative. This state of the skin persisted for 48 hours. Two days later the animal once more reacted to the intradermal injection of the testing fluid, giving a rise of 4 mm. (positive).



Graph 7.—W.B.C. response to injection i/v of 0·7 g. sphingomyelin on two consecutive days. Lymphocytes are depressed on both days but the neutrophils rise only on the second day. The skin is desensitised.



Graph 8.—W.B.C. response to i/v injection of 1·0 g. sphingomyelin. Cross-over of lymphocytes and neutrophils. Animal is desensitised. (D4.)

Examples of desensitisation with sphingomyelin showing these two types of blood reaction were obtained.

D4, a sensitised non-gravid cow.

19.10.48: The skin reaction rose to 5·5 mm. (positive). On 20.10.48, seven hours after an intravenous injection of 1 gramme of sphingomyelin the skin reaction rose to 5 mm. (positive); 24 hours later it was 2 mm. (negative).

Graph 8 shows a typical crossover with a very marked depression of the lymphocyte number and rise of the neutrophils. At 8½ hours the lymphocytes were still low. The normal conditions had re-established themselves in 24 hours.

Three sensitised animals, E3, E6 and E10, were all injected intravenously with 1·0 gramme of sphingomyelin from one batch. The injections took place on the same day. They were all desensitised and all showed a marked depression of the lymphocyte count without a rise in the number of neutrophils.

The desensitisation results were as follows :—

E3; sensitised calved heifer.

6.10.48: Skin reaction showed a rise of 5 mm. (positive).

7.10.48: Injection of sphingomyelin; eight hours later the skin showed a rise of 1 mm. (negative); 8.10.48, 10 a.m.: the skin showed a rise of 3·5 mm. (low positive); 9.10.48, 11 a.m.: the skin showed a rise of 6 mm. (positive).

In the case of E6 the course of the desensitisation was as follows :

E6; sensitised calved heifer.

6.10.48, 11 a.m.: The skin showed a rise of 7 mm. (positive).

7.10.48: Injection of Sphingomyelin; eight hours after injection the skin showed a rise of 4·5 mm. (positive); 8.10.48, 24 hours after injection: the skin showed a rise of 2 mm. (negative); 9.10.48, 11 a.m., 50 hours after injection: The skin showed a rise of 5·5 mm. (positive).

E10, a sensitised bullock, was also desensitised.

6.10.48, 11 a.m.: The skin showed a rise of 4 mm. (positive).

7.10.48: Injection of sphingomyelin; eight hours later the skin showed a rise of 1 mm. (negative); 8.10.48, 24 hours after injection, the skin showed a rise of 1·5 mm.; 9.10.48, 11 a.m., 48 hours after injection, the skin showed a rise of 4 mm. (positive).

The course of the white blood corpuscle reaction in E10 is shown in Graph 9. The graphs of the changes in E6 and E3 are so similar that it is not necessary to reproduce them. It will be seen that before the injection, the lymphocytes were slightly above the 5,000 level with neutrophils just below 3,000, the total count being 8,800. The total count dropped continuously until at the fifth hour it was only 5,400; after that it rose steadily until at 24 hours it was once more 8,800. The neutrophils showed a dip half an hour after the injection, but regained the pre-injection level an hour later and thereafter they were little affected. The lymphocyte number dropped steadily and reached the low figure of 2,160 at five hours; they were still low, though rising a little at eight hours (3,150). At 11½ hours after injection the lymphocytes had risen and now numbered 4,800. In

GRAPH 9.—W.B.C. response to 1 g. sphingomyelin i/v. Lymphocytes are depressed in number but neutrophils do not rise. The skin is desensitised. (E.10.)

24 hours the W.B.C. count showed the pre-injection relations. The noticeable feature in all these animals is the depression of the lymphocytes and the failure of the neutrophils to rise.

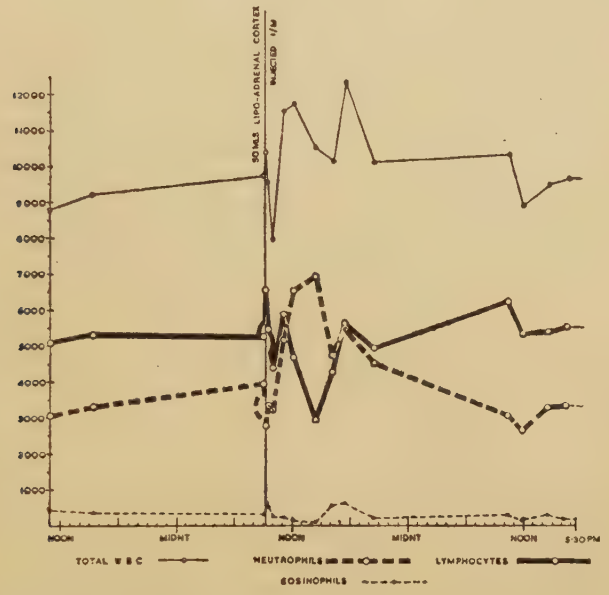
It should be noted that it is characteristic of the reaction to sphingomyelin that the desensitisation is of short duration and in these three examples the skin sensitivity had re-established itself 48 hours efter the injection.

Adrenal-cortical Extract.—The substance used was the lipo-adrenal cortex preparation of Upjohn, which contains all the known active hormones which have an oxygen atom attached at C11. It should be noted that the desoxycortecosterone compounds were found to be without action on the lymphocytes by Dougherty and White (1947) and by Hills, Forsham and Finch (1948).

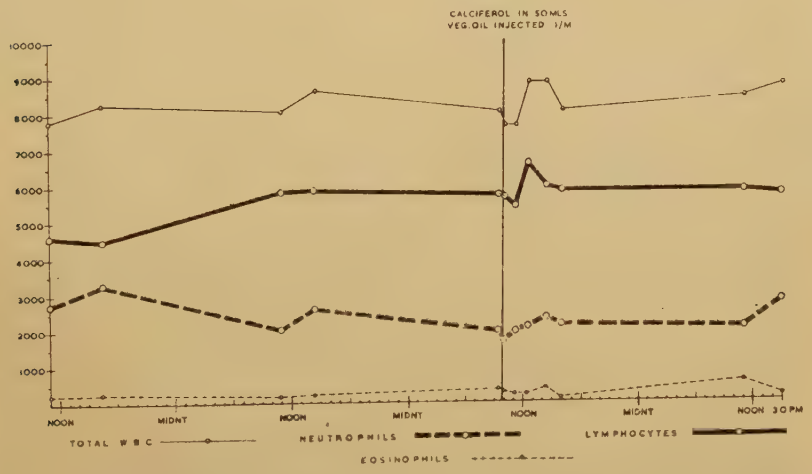
Our supply of the adrenal-cortical steroid solution was strictly limited, and we were unable to experiment with it with any freedom. It was used in the first place to confirm in the bovine the action on the white blood corpuscles noted by the workers, cited above, who used rabbits, mice and man.

A total dose of 50 ml. was divided and injected at two sites into a non-gravid cow. The effect on the differential leucocyte count is shown in Graph 10. The characteristic drop in the lymphocytes and the rise in the neutrophils became marked after the second hour and the peak of the reaction was at $5\frac{1}{2}$ hours. At seven hours the lymphocyte number was rising again and the effect of the injection was obviously wearing off. In 24 hours the normal relations between the lymphocytes and the neutrophils had been re-established. Graph 11 shows the effect on the W.B.C. counts of the injection of 50 ml. arachnis oil containing a large dose of calciferol. There was no change in the relation of the various elements. We were only

able to do one more experiment with the adrenal-cortical preparation. A virgin heifer, E8, was vaccinated by intramuscular injection of acetone-dried trichomonas antigen (strain Manley). The last



GRAPH 10.—W.B.C. response to i/m injection of 50 ml. of adrenal-cortical steroid. Cross-over of lymphocytes and neutrophils. Peak of reaction is at 6 hours.



GRAPH 11.—W.B.C. counts in animal receiving 50 ml. i/m of arachnis oil containing calciferol.

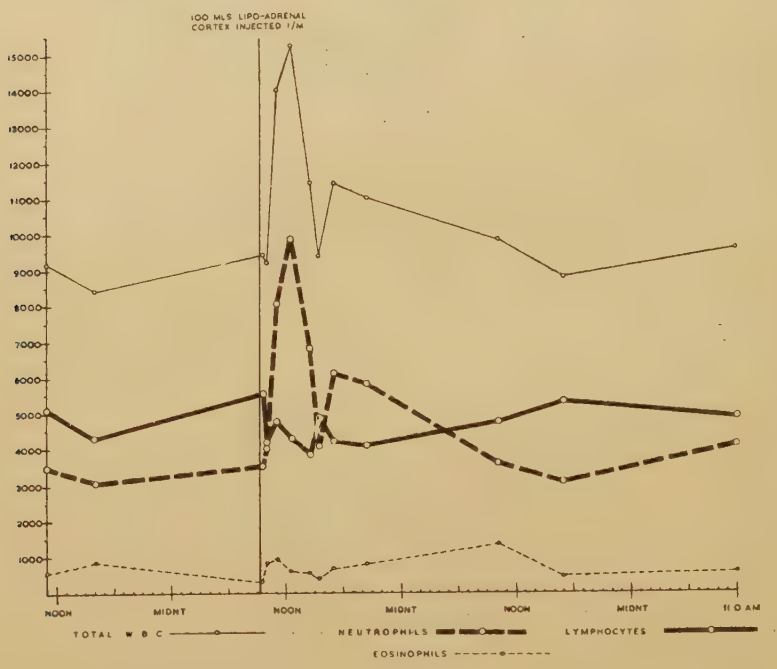
vaccinating dose was given on 14.9.48, 23 days before the injection of the hormone. The effect upon the skin reaction was as follows :

E8

6.10.48 : The skin showed a rise of 8 mm. (high positive).

7.10.48, 9.30 a.m. : 100 ml. of lipo-adrenal-cortical preparation injected intramuscularly distributed between four sites ; seven and a half hours after injection, the skin showed a rise of 3·5 mm. (low positive) ; 8.10.48, 24 hours after injection, the skin showed a rise of 4·5 (positive) ; 9.10.48, 48 hours after injection, the skin showed a rise of 7 mm. (high positive).

The desensitisation was not quite complete though the loss in sensitivity was well marked. The return to the original order of reaction in 48 hours was similar to that observed with sphingomyelin. The total W.B.C. count (Graph 12) rose steeply and there was a very marked increase in the neutrophils. The lymphocyte count was



GRAPH 12.—W.B.C. response to 100 ml. adrenal cortical steroid i/m. The lymphocytes are moderately depressed for 10 hours. Neutrophils rise steeply. The skin reaction is very much reduced for about 24 hours. Return to pre-injection degree of reaction in 48 hours.

depressed, and while the dip is not so obvious as in some of the graphs obtained during this work on depression, it was of considerable duration lasting at least ten hours. On the day after the injection the effect of the hormone on the leucocyte count had disappeared. The same animal (E8) was given 100 ml. of arachnis oil two weeks

later. There was no effect upon the W.B.C. relations except a slight rise in the total count. There was no desensitisation of the skin. The injection of 30 mg. of stilboestral propionate (E6) was also without effect upon the W.B.C. differential count and the skin reaction was not reduced.

DISCUSSION

In this work all the cases of desensitisation by absorption of antigen seem to present the same characteristics. There is a quantitative element, that is to say that if the dose of antigen administered is too small, desensitisation does not occur. But a mere quantitative basis of neutralisation of antibody throughout the body of the animal does not afford a satisfactory explanation since one of the features is the failure appreciably to reduce the titre of the blood. A very striking example is the absorption of antigen in those cases where the uterus is filled with living trichomonas since the skin may be negative for a considerable period while the blood titre is very high. One cannot neglect the possibility that circulating antigen might reduce the skin response on the analogy that all reactions with fixed or localised antibody are diminished so long as there is unconverted antigen still present (Weil, 1914; Denzer, 1916).

It would appear that the general physiological effect must be taken into account. We have found that the absorption of antigen desensitises the skin in a large proportion of cases but quite certainly not in all. In animals which have had high blood titres for prolonged periods and where the skin reaction has been very marked, it is much more difficult to bring about desensitisation: moreover, in some instances we have not been able to desensitise these animals with the doses used. The cases where desensitisation does not occur require special study but it would appear that the general physiological response was insufficient or the degree of fixation was not alike in all.

In these experiments, whenever trichomonas-sensitive animals were tested before and after parturition, the skin was found to have been desensitised. This does not invariably take place, however, in tuberculin-sensitive animals (Kerr, Lamont and McGirr, 1946), and we do not suppose it would occur in all cases of animals sensitive to trichomonas antigen. Our studies of the W.B.C. counts during the parturition period of animals calving normally show interesting variations which may account for this. In parenthesis, it may be said that while there is a fundamental parallelism between the desensitisation at parturition in tuberculin-sensitive animals and in trichomonas-sensitive ones, we do not consider the process to be necessarily identical in detail. The type of skin reaction in the two cases show certain differences and these two examples of sensitivity must be studied independently.

In all cases of desensitisation where counts were made the blood picture has shown a change in the lymphocyte/neutrophil ratio.

The character of the white cell picture is the depression of the lymphocytes accompanied by a greater or lesser run up of the neutrophils. The desensitisation graphs are all of a very similar type, and it would appear that there is a correlation between the depression of the lymphocytes and the desensitisation.

This reaction on the part of the white blood corpuscles is well recognised. It has been described as a result of the action of cortical extract and adrenotrophic hormone in normal animals (Hills *et al.*, 1948; Dougherty and White, 1947). It has been found also in the hormone-like action of injected sphingomyelin (Tompkins, 1946). We have shown it occurring after the injection of antigen into animals already basically immunised (Graphs 2 and 3). This supports the work of Chase *et al.* (1946), who concluded that the stimulus to antibody production is connected with adrenal cortical activity. In parturition, while the exact interaction of the hormones and the sequence in which they come into play are not clearly understood, it is generally accepted that at the end of pregnancy, and at parturition, there is a change in the hormonic balance (Robson, 1947; Dukes, 1943).

The different species of animals appear to show considerable difference in detail, and we are inclined to think W.B.C. graphs in parturition may differ somewhat even in the same species according to the hormonic pattern of the individual. All that can be done here is to point out the characteristic W.B.C. graph (Graph 4) and to suggest that we have a type of underlying physiological reaction of which one effect is the production of desensitisation. Our view is that the white cell picture in all these cases is related to the mobilisation of the pituitary cortical hormone complex and the desensitisation follows as one of the results.

These observations of the W.B.C. reaction in bovines to the injection of antigen do not stand alone. The response of the blood cells to injection of antigen in tuberculin-sensitive animals has been studied by Hendershott and Johnston (1941), who found a reduction of the lymphocyte/neutrophil ratio and some degree of drop in the absolute number of lymphocytes, as in the case of trichomonas-sensitive animals observed by the present writers. This work has been confirmed in its broad outline by Young, Paterson and Mastin (1944),* who found the crossing over of lymphocytes and neutrophils in infected animals upon subcutaneous injection of "whole human tuberculin."

Kerr *et al.* (1949), using two intradermal injections of tuberculin administered into the same site one week apart, also observed the cross-over reaction between neutrophils and lymphocytes in animals infected with tuberculosis. The infection was verified at slaughter, thus amply confirming Hendershott and Johnston's results. Negative animals treated in exactly the same way gave no such response.

* Unpublished Report to the Agricultural Research Council, 1944.

In our work on trichomonas the bleb formation was used as the criterion of the state of fixation of antibody or sensitiveness of the skin. The exact event in the skin remains obscure in spite of the large amount of research done on this type of reaction (Ratner, 1943). It seems, however, to be generally agreed that lymph or tissue fluid is exuded into or collected at the site of the disturbance caused by the antibody-antigen junction and that the fluid is retained there for a sufficient period of time to produce the bleb. The permeability of the capillaries is capable of considerable variation (Landis, 1946), and while the capillary wall under normal conditions is an efficient barrier to the passage of protein the response in urticarial lesions shows that protein does in these conditions pass into the oedema fluid. The temporary retention of the fluid may be due to this.

The intradermal test in this work is partially at all events an introduction of hapten into the lymph spaces of the skin, and it seems highly probable that the lymph and perhaps the lymphocytes take part in the formation of the bleb. The exact mechanism of the connection of the lymphopenia and the temporary desensitisation of the skin (*i.e.*, failure to react) is not clear and it would be premature to attempt to draw detailed conclusions. Nevertheless, as the lymphocyte appears to have, at least as one of its functions, the carrying of globulin through the lymph spaces to all parts of the body and as further the lymphoid tissue is to a considerable extent under the control of the adrenal cortical hormone and of the pituitary adrenotrophic hormone which are instrumental in its production (Ehrich and Harris, 1946 ; White and Dougherty, 1946), it does not seem to be unreasonable to consider that the desensitisation is connected with the lymphopenia.

The standpoint of the present writers is that from the available evidence desensitisation, as shown in the reaction to the injection of antigen and as a response to adrenal-cortical hormone and sphingomyelin, is a failure to react by bleb formation rather than a denuding of fixed antibody from the skin. This is also probable, but is not quite so clear in the more complex conditions of parturition where desensitisation is of longer duration. Certainly the failure to react appears to have as a closely accompanying character the white blood corpuscle reaction and the lymphopenia described above.

SUMMARY

The desensitisation of the skin in bovines which have been sensitised to Trichomonas antigen, has been studied.

Desensitisation has been found to take place upon the absorption of further antigen in appropriate doses in the following circumstances : (1) from intramuscular injection of antigen ; (2) from absorption of antigen in acute infections from the breakdown of trichomonads proliferating in the uterus in large numbers ; (3) from instillation of antigen into the uterus of non-gravid cows.

Non-specific desensitisation of the skin has been observed at parturition and upon injection of C—11 oxygenated adrenal-cortical hormone and after injection of sphingomyelin.

The changes in the white blood corpuscle counts have been observed in selected examples, and the correlation between the characteristic cell reactions produced and the desensitisation is discussed.

ACKNOWLEDGMENTS

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